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THE UNIVERSITY OF ALBERTA

STUDIES OF THE PROTEINS AND AMINO ACIDS IN THE POTATO

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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External Examiner

Date June 15 / 1979

DEDICATED TO THE MEMORY
OF MY GRANDPARENTS AND PARTS OF THE FAMILY
WHO LOST THEIR LIVES DURING THE SECOND WORLD WAR

AVIGDOR and CILA KIZBER

and

YEHUDA ARIE and SHEINDLE GOLDHIRSH

ABSTRACT

Potato proteins and amino acids were analyzed by several physicochemical methods. A gas chromatographic procedure was developed for amino acid analysis using the N-heptafluorobutyryl-isopropyl ester derivatives. Qualitative and quantitative analysis was obtained within 11-14 min. A glass column connector for a gas chromatograph was developed while setting-up the analytical system. The combination of gas chromatograph-mass spectrometer enabled direct identification of γ-aminobutyric acid (GABA) and ornithine derivatives in a free amino acids (FAA) extract of the potato. A procedure for direct glutamine and asparagine (amides) determination in the form of their corresponding amino acids in FAA extract was developed.

The nitrogenous compounds of the potato tuber ('Netted Gem') were found mostly in a dialyzable form (>50%). The non-dialyzable nitrogen (protein) consisted of approximately 50% albumins, 30% globulins, 16% basic glutelins and the rest were prolamines and acidic glutelins.

Thin layer isoelectric focusing (TLIF) on granulated gel showed 8 and 13 protein bands in the water insoluble and soluble fractions, respectively. The major protein bands in the water insoluble fraction had high isoelectric points in the pH 8.5 region. The water soluble fraction had a wider range of pHs (4.5-8.5). Amino acid composition of both fractions was similar, except for slightly higher content of basic amino acids in the water insoluble fraction. The major protein subunits obtained by SDS,DTT treatment were in the low molecular weight range (10,000 and 30,000).

The outer layer (OL) of the tuber had significantly higher (2.2%) content of nitrogen than the inner layer (IL) (1.6%). The proteins pattern, according to solubility and TLIF, was similar in both parts of the tuber. The OL of the tuber contained a higher amount (150.11 mg/g) of amino acids than the IL (83.96 mg/g). Aspartic acid was exceptionally high (50.24 mg/g) in the OL of the tuber. Percent 'essential amino acids' (% EAA) was slightly higher (38.3%) in the OL than in the IL (35.3%). An increase in nitrogen content was found in 'Netted Gem' tubers during growth on a fresh weight basis. There were differences in nitrogen content among potato cultivars tested, on a dry weight basis only. The pattern of amino acid composition in the different cultivars was similar. The major amino acids were aspartic and glutamic acids (including the corresponding amides). Methionine was detected in the smallest quantities. % EAA was similar in mature tubers of the different cultivars (average 38.2%). It decreased during 'Netted Gem' tuber development.

There were significant losses in total potato nitrogen and FAA during the add-back process (35-40%). The major losses occurred at the pre-cooking and mash-mixing steps. The FAA pool of the potato was composed of protein amino acids and in addition GABA, α-aminobutyric acid and ornithine were detected by gas chromatographic analysis. The amides and corresponding amino acids comprised the major part (53.5%) of the FAA pool of the potato.

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ABBREVIATIONS

AA - Amino Acid

A-B - Add-Back

DTT - Dithiothreitol

FAA - Free Amino Acids

GC - Gas Chromatograph

GC-MS - Gas Chromatograph-Mass Spectrometer

GLC - Gas-Liquid Chromatography

HFB - Heptafluorobutyryl

HFBA - Heptafluorobutyric Anhydride

IEC - Ion-Exchange Chromatography

IL - Inner Layer

NPN - Non-Protein Nitrogen

OL - Outer Layer

PAGE - Polyacrylamide Gel Electrophoresis

RMR - Relative Molar Response

RRT - Relative Retention Time

RT - Retention Time

SDS - Sodium Dodecyl Sulfate

TAB - N-Trifluoroacetyl-*n*-Butyl Ester

TFA - Trifluoroacetyl

TFAA - Trifluoroacetic Anhydride

TLIF - Thin Layer Isoelectric Focusing

1. INTRODUCTION

The potato is an important food crop (Van der Zaag, 1976). Usually it has not been considered as a source of protein because of the low protein content, approximately 2.1% on a fresh weight basis. This protein represents 10.4% of the total solids of the potato (Markakis, 1975). Three decades ago, only two main proteins were thought to occur in the potato tuber. They were called tuberin and tuberinin. In a small sample of potato sap it is now possible to detect many proteins by electrophoretic techniques. New techniques for proteins separation have provided tools for better resolution of protein fractions as a first step in their characterization. The major research in potato proteins was directed to identification of cultivars by electrophoretic techniques (Stegemann *et al.*, 1973). Little is known about the diversity of potato proteins and their physicochemical properties. Quantitative studies on the whole tuber proteins and amino acids have shown a large variability in composition among cultivars, location of growth and conditions of growth. Therefore, it was of primary importance to determine the nitrogen content of Alberta grown potatoes used in this study, as basic background for further characterization. The nitrogen distribution within the individual tuber was considered in only a limited number of reports. In this research, special attention was given to the nitrogen and proteins composition in the outer (peel) and inner layers of the tuber. It is generally accepted that a large proportion of potato nitrogen is in a non-protein nitrogen (NPN) form. This fraction contains large amounts of amides (Steward and Street, 1946). The amides have a significant role in determining the physicochemical properties of plant proteins (Stegemann, 1975). Therefore, the free amino acid pool of the potato was studied and special consideration was given to its amide content.

As nutritional status of processed products is now being stressed, along with the economic, nutritional and environmental impacts of processing technology, the nitrogen and amino acids content of the potato were considered in relation to the add-back process for potato granules production.

In order to cope with the variety of subjects under consideration, different physicochemical methods of analysis were used and others were developed as the work progressed.

2. LITERATURE REVIEW

2.1. *The Potato Tuber*

Potato tuber (*Solanum tuberosum* L.) is morphologically a modified stem. A schematic longitudinal section of a tuber (Figure 1) shows two zones of tissues - a central pith with its lateral branches, and the vascular ring surrounded on either side by storage parenchyma. The outermost layer ('skin') of a mature tuber is formed by the periderm, a layer of cork cells (Fedec *et al.*, 1977). The pith forms the central part of the tuber. The cortex forms a narrow band of tissue limited internally by a zone of external phloem, a region of disconnected vascular bundles, and externally by the periderm. In this work, the inner layer (IL) refers mostly to the pith and storage parenchyma on the inner side of the vascular ring. The outer layer (OL) or peel refers mostly to the outer layer of the tuber limited on the inside by the vascular ring (Figure 1). Where not otherwise specified, peeled potato is assumed.

The potato tuber is mainly a storage tissue for starch, although protein crystals resembling storage proteins in seeds were observed in early anatomical studies of the potato (Artschwager, 1924). These crystals appear during the initial stages of tuber development in the OL. In the mature tuber more than one may be observed in a cell. Recently, they were isolated and characterized (Hoff *et al.*, 1972). However, the most extensively characterized proteins of potato tuber are the protease inhibitors which represent only 10% of total tuber proteins (Ryan, 1973). This group of proteins was used in a few

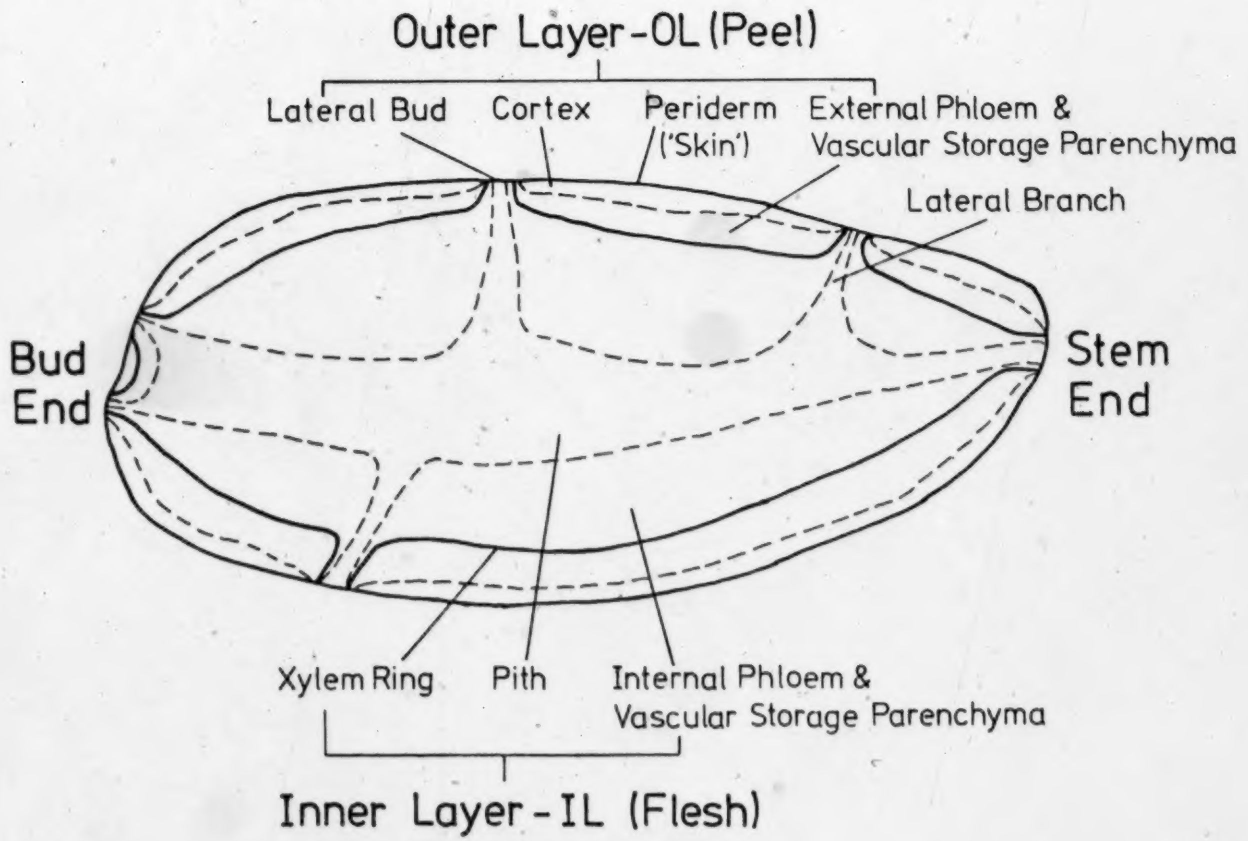

Outer Layer-OL (Peel)
Lateral Bud
Cortex
Periderm ('Skin')
External Phloem & Vascular Storage Parenchyma
Lateral Branch
Bud End
Stem End
Xylem Ring
Pith
Internal Phloem & Vascular Storage Parenchyma
Inner Layer-IL (Flesh)

Figure 1. Schematic longitudinal section of a potato tuber.

studies for identification of potato cultivars (Kaiser *et al.*, 1974; Ryan and Pearce, 1978).

Total nitrogen content of the potato is affected by many factors; cultivar, conditions of growth, fertilizer levels, and storage conditions. Therefore, a wide range of values for protein content of the tuber is found in the literature. In a proximate analysis of white potatoes, Talburt *et al.* (1975) reported a range of 0.7-4.6% for proteins.

The protein concentration in young tubers was shown to be twice as high as in mature ones (Zimmermann and Rosenstock, 1976). They also found that protein concentration decreased during development to a fairly constant level of approximately 65 mg/g once the tuber has reached a size of 2-3 centimetres in diameter. During long periods of storage of mature tubers at a temperature of 7°C, there was no significant alteration in protein level. A loss of 3% of the tuber proteins during six months of storage at about 3°C was found by Desborough and Weiser (1974). An inverse relationship between total and 70% ethanol soluble nitrogen to total solids of potato tubers stored at 3°C for 10 months was calculated on a dry weight basis (Talley *et al.*, 1961). But, when nitrogen content was expressed on a fresh weight basis, no significant difference was found between samples of different solids contents. This indicates that the apparent inverse relationship between protein and solids, on a dry weight basis, is probably due to changes in other constituents of the dry matter of the tuber and that the nitrogen content is not affected upon storage.

Mineral fertilization was shown by Mulder and Bakema (1956) to increase the total nitrogen content of the tuber, but amino acid composition of the proteins was not affected. The increase in total nitrogen was reflected in higher content of soluble non-protein nitrogen (NPN), mainly in the form of amides. Under high nitrogen fertilization the glutamine content was increased even more than that of asparagine. A similar effect of nitrogen fertilization was reported recently by Rexen (1976). An additional effect observed was a significant decrease in protein quality accompanying the increase in total nitrogen content. Large differences in the response to nitrogen fertilization was found among cultivars.

Protein and amino acid distribution within a single tuber could have contributed to the variability in nitrogen values reported. Only a limited number of reports on this aspect of potato nitrogen is available. Cotrufo and Levitt (1958) have reported on changes in the ratio of 80% ethanol soluble and insoluble fractions in relation to the external (OL) and internal (IL) layers of the tuber. They suggested that the variations in the relative amounts of these fractions in the different parts of the tuber are related to different physiological states. High amino acid content in the internal tissues was associated with deep rest, high protein with the end of the rest period, while the changes in the OL were opposite to that of the IL. Cotrufo and Levitt's report indicated that it is important to investigate both parts of the tuber. However, their observations were overlooked for many years. Johnston *et al.* (1968) analyzed different parts of the tuber in relation to nitrogen content. They found higher nitrogen content in the OL than in the pith. Schuphan (1970) reported that the

OL (mainly the cortex) of the tuber had a much higher concentration of essential amino acids than the IL. This observation lead that group to take this phenomenon into account in breeding potatoes containing protein of high biological value, i.e. by increasing the OL in proportion to the whole tuber. Desborough and Weiser (1974) found no significant differences in protein content among the cortical, medullary and pith regions of the tuber. Recently, Weaver *et al.* (1978) found significant differences between the core of the tuber, the bud-end and the stem-end with regard to total nitrogen and free amino acids. Core tissue in all cultivars tested had more total nitrogen than did tissue at either end of the tuber, while the total nitrogen content was similar at both ends. In contrast to these findings, Johnston *et al.* (1968) reported that the stem-end contained more total nitrogen than did the bud-end. These controversial reports indicate the need for further research to clarify these aspects of tuber composition.

2.2. *Quantitative Determination of Potato Proteins*

2.2.1. Protein Determination

The common methods for protein determination, which are widely used in potato protein analysis are: 1. Kjeldahl-N determination used to calculate total (or crude) potato proteins using 6.25 as the conversion factor (Desborough and Weiser (1974) reported that potato proteins contain about 13.5% nitrogen and suggested 7.4 as a more appropriate conversion factor). 2. The Folin-Ciocalteau reagent is used in the procedure developed by Lowry *et al.* (1951) (Lowry's method) or modifications thereof (Potty, 1969). 3. The Biuret method is widely used (Bailey, 1967). It was adapted for determination of the heat coagulable proteins of the potato by Van Gelder and Krechting (1973). 4. In many cases, protein fractionation by column chromatography as well as protein isolation by other physicochemical methods are followed by spectrophotometric measurement of protein solutions at 280 nm. 5. A reliable estimate of protein content is obtainable from total amino acids recovered by amino acid analysis on ion-exchange chromatography. The use of any one of these methods depends on the amount of protein and the sensitivity of the method.

Several studies have been directed towards the adaptation of general protein determination methods to potato proteins. When the Kjeldahl method is used for potato nitrogen determination, it must be taken into account that the calculated protein value does not give a true protein estimate because most of the organic nitrogen is included in nitrogen measured by this method (Bradstreet, 1965). The organic nitrogen of the potato includes, in addition to the proteins, free amino acids, amides and basic nitrogen compounds (Woodward and Talley, 1953). In order to obtain a true protein nitrogen estimate by the Kjeldahl method, a protein extract has to be dialyzed or otherwise purified in order to remove non-protein nitrogen.

Rapid methods for potato protein determination have been mainly used in breeding programs, where many samples have to be analyzed. Dye-binding reactions based on the binding of ionic dyes (Amido-black, Orange G and Bromophenol blue) to protein to form insoluble complexes, have been used. The excess dye left in the solution is measured spectrophotometrically, and correlated with total protein content measured by an established method (usually Kjeldahl). Kaldy *et al.*

(1972) obtained high correlation coefficient (r = +0.9827) between the Orange G dye method and Kjeldahl nitrogen determination. Desborough (1975), comparing a few methods of protein determination, found that Bromophenol blue correlated well with the routine methods (micro-Kjeldahl, Lowry's method as modified by Potty (1969) and amino acid analyses). A refractometric determination of soluble proteins in expressed potato juice was shown to correlate well with Lowry's method (Hoff, 1975). An alkali-phenol method was recently adapted for potato protein determination by Mohyuddin and Mazza (1978). The Neotec Grain Quality Analyzer was evaluated for use in potato protein determination, but no consistent results were obtained when Neotec protein readings were compared with other methods (Desborough, 1975).

2.2.2. Protein Solubility

Globular proteins vary considerably in their solubility in aqueous solutions. These differences may be used to bring about separation of mixtures of proteins. The major variables affecting the solubility of proteins are: pH, ionic strength, the dielectric properties of the solvent and temperature (Lehninger, 1975).

Osborne's studies in the early 1900's resulted in the classification of plant proteins on the basis of their solubilities in various aqueous solutions (Osborne, 1908). This classification is still widely used. Proteins soluble in water are referred to as albumins; those insoluble in water, but soluble in dilute salt solutions are called globulins; those insoluble in the above solutions but soluble in weak acidic or basic solutions are the glutelins; those insoluble in the above solutions but soluble in 70 to 80% ethanol are the prolamines

(Ashton, 1976). This simple classification scheme is followed in the work reported in this thesis.

The fractionation of potato proteins according to solubility has been reported by several workers. Among the earliest research reported was the report by Osborne and Campbell (1896). They performed salting-out experiments and reported that the protein of the potato was mainly a globulin and named it tuberin. In more recent work, Groot *et al.* (1947) isolated two major and one minor protein fractions from the potato. The new major fraction was called tuberinin; it was characterized as more hydrophilic and having lower isoelectric point than tuberin. Two-thirds of the potato proteins were extracted in a dilute salt solution by Levitt (1951). His crude protein extract was fractionated into almost equal quantities of albumins and globulins. The albumins were separated into acid soluble and insoluble fractions. Further fractionation of potato proteins was achieved by Lindner *et al.* (1960). They obtained 4.0% albumins, 77.8% globulins, classified into slightly and readily soluble fractions (1.4 and 76.4%, respectively), 1.8% prolamines, 5.5% basic glutelins and a non-soluble fraction (10.9%). The potato proteins extracted by a phosphate buffer containing 0.1M NaCl were separated into equal amounts of albumins and globulins using dialysis and each fraction was further characterized by other physico-chemical methods (Nakasone *et al.*, 1972). Most recently Kapoor *et al.* (1975a) reported two different solubility profiles of potato proteins, obtained by different procedures. One procedure was based on the method of fractionation of Lindner *et al.* (1960) and the other one was based on the method of Nagy *et al.* (1941). Both methods yielded high amounts of albumins and globulins. The combined totals for albumins and globulins were 81% for Lindner's method and 75% for Nagy's method. As well 0.2% NaOH soluble glutelins were 7.6% and 8.8%, respectively, for the two methods. The rest were prolamines and a non-soluble residue. However, the relative amounts of albumins and globulins were not in good agreement. In the first method, 6.6% albumins and 74% globulins were found, while in the second method 49% albumins and 26% globulins were obtained. The differences were attributed to the possible extraction of albumins, in large quantity (~40%) in the readily soluble fraction of the globulins. These results indicate the somewhat arbitrary nature of fractionation according to solubility.

2.3. *Electrophoretic Patterns of Potato Proteins*

2.3.1. Electrophoresis

Electrophoresis refers to the movement of charged macromolecules under the influence of an electric field. Depending upon their net charge, macromolecules migrate either to the cathode or anode. Differences in migration velocities provide a sensitive analytical property for the separation and identification of proteins. A wide selection of substances are available as support media for the stabilization of the bands of separated protein. Currently one widely used support is the polyacrylamide gel. The use of polyacrylamide gel electrophoresis (PAGE) was first described in detail by Ornstein (1964) and Davis (1964). The potential of PAGE can be very much increased by using different gel concentrations, degree of crosslinks, or by introducing reagents with specific effects such as urea and sodium-dodecyl sulfate (SDS, which will be discussed later) (Chrambach and

and Rodbard, 1971).

The fact that proteins cease to migrate in an electric field, at their isoelectric points, is exploited in isoelectric focusing in polyacrylamide gel, or in granulated gels (Sephadex). The principle of isoelectric focusing is illustrated schematically in Figure 2. A stable pH gradient increasing from anode to cathode is established by electrolysis of carrier ampholytes in the anticonvective medium. Any amphoteric compound subjected to the pH gradient will be charged according to its isoelectric point (IP), and will migrate towards the pH of its isoelectric point, where the net charge on the molecule will be zero. The use of thin layer isoelectric focusing (TLIF) on granulated gel has been shown to be useful and technically easier for analytical as well as preparative purposes (Radola; 1973, 1974).

2.3.2. Electrophoresis of Potato Proteins

As early as 1947, Groot *et al.* have shown the presence of tuberin and tuberinin using paper electrophoresis. Yasuda *et al.* (1955) obtained 6 protein fractions in a potato extract by paper electrophoresis. Paper electrophoresis was utilized for characterization of 59 Dutch potato cultivars (Zwartz, 1966). However, no effect on mature potato protein patterns within a cultivar was found due to soil type, fertilization or environmental conditions. Similar results were also obtained on PAGE by Zacharius *et al.* (1971), Zimmermann and Rosenstock (1976) and others. These findings justified the use of PAGE patterns for cultivar identification. It has been used extensively by several research groups (Desborough and Peloquin, 1966; Stegemann *et al.*, 1973; Kaiser *et al.*, 1974). Identification of potato cultivars based on PAGE pattern of specific enzymes was also used (Desborough and Peloquin, 1968).

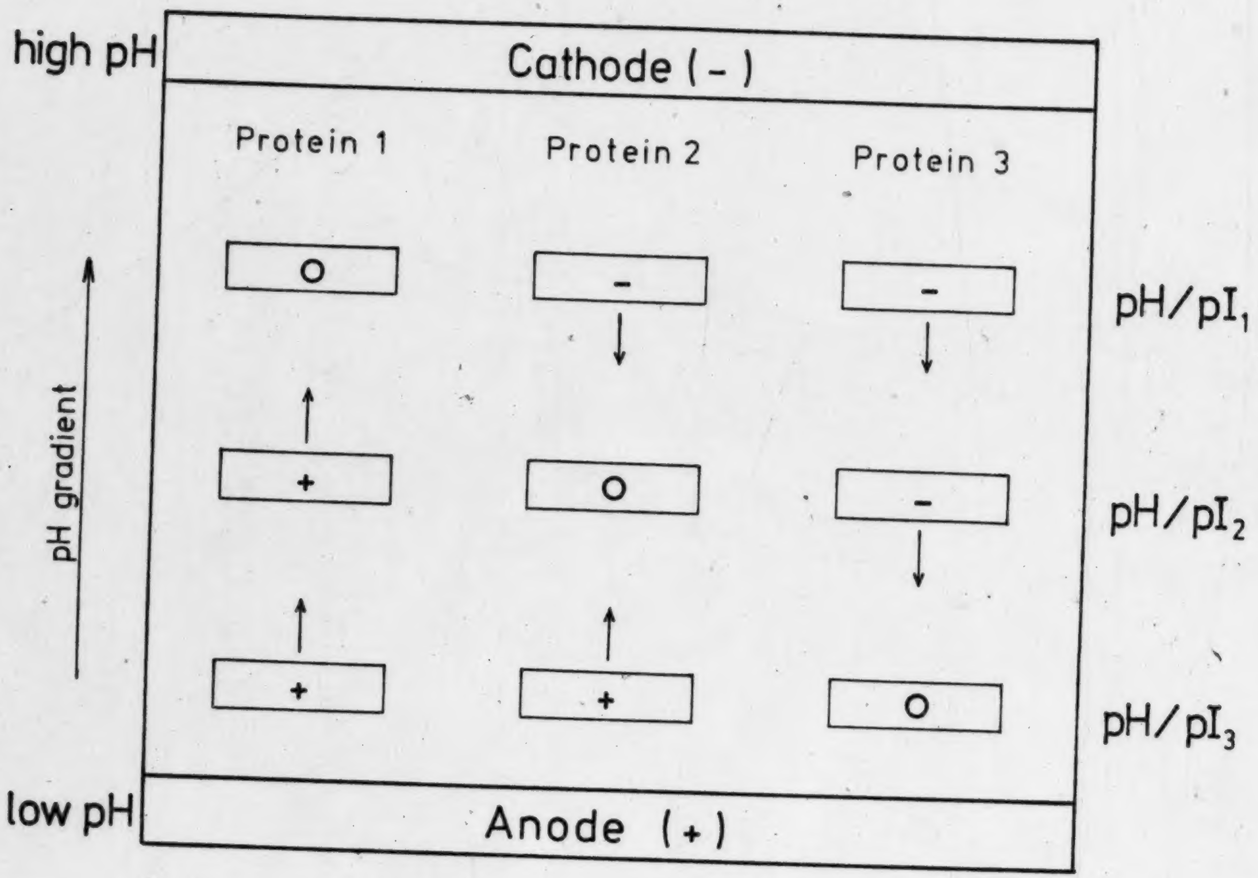

high pH
Cathode (-)
Protein 1
Protein 2
Protein 3
pH gradient
O
-
-
+
O
-
+
+
O
pH/pI$_1$
pH/pI$_2$
pH/pI$_3$
low pH
Anode (+)

Figure 2. Schematic representation of proteins separation by isoelectric focusing. Proteins 1, 2 and 3, with isoelectric points pI_1, pI_2 and pI_3, respectively, focused on a thin layer.

By employing enzyme systems instead of protein systems, no destaining of the gels is necessary, and the resultant patterns have fewer bands and thus are easier to compare. However, when enzyme patterns from two or more cultivars are similar, then protein comparisons are necessary. The enzyme systems employed were polyphenol oxidase (Nye *et al.*, 1968), esterase and peroxidase (Desborough and Peloquin, 1968 and 1969), peroxidase and malate dehydrogenase (Zimmermann and Rosenstock, 1976). Trypsin and chymotrypsin inhibitors were employed by Kaiser *et al.* (1974). Reviews of this subject were given recently by Stegemann *et al.* (1973) and Stegemann (1975).

2.4. *Protein Molecular Weight Determination*

There are several methods for protein molecular weight determination, but only those used in this work will be briefly presented.

2.4.1. Molecular Weight Estimation by Gel Filtration and SDS-Gel Electrophoresis

Gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) are widely used for molecular weight estimation of proteins. They share one fundamental feature; the "molecular sieving" effect of the gel. The most commonly used gels are crosslinked dextrans (Sephadex) and polyacrylamide, respectively (Rodbard, 1976). The common practice is to construct a calibration curve using proteins of known molecular weight, then under the same conditions analyze the unknown mixture and obtain the estimate of molecular weights of the mixture components by comparison with the standard curve.

Different theories have evolved to correlate the molecular weight and size of a macromolecule to its mobility in the gel medium (Rodbard, 1976). In gel filtration an empirical logarithmic relationship was found between the partition coefficient (Kav) and molecular weight (MW).

$$Kav = -A \log MW + B$$

where: A and B are empirical constants.

Kav is obtained from the following equation:

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

where: V_e = elution volume
V_o = void volume
V_t = total bed volume

A similar relationship between protein mobility in SDS-PAGE and molecular weight was first indicated by the results of Shapiro *et al.* (1967). It was confirmed and extended by Weber and Osborn (1969). The interaction between SDS, which has a strong anionic end group, and proteins results ideally in a uniform charge density on all proteins in a mixture. This makes the separation on PAGE dependent mostly on the molecular size which is analogous to the situation in gel filtration (Rodbard, 1976). Assuming that the crosslinking and shape of the gel are constant, the relative mobility of the molecule will be a direct function of its molecular weight. Hence, the relationship between molecular weight (MW) and mobility in SDS-PAGE can be expressed by the equation below (Shapiro *et al.*, 1967).

$$MW = K\,(10^{-bx})$$

where x is the relative mobility, b is the slope of the line and K is a coefficient, characteristic of the gel medium (retardation coefficient). It can be also expressed as a plot of log of molecular weight versus relative mobility.

2.4.2. Molecular Weight Distribution of Potato Proteins

The use of gel filtration in potato protein studies was mainly for the purification of specific enzymes and for removal of small molecular weight material in extraction procedures (Loeschcke and Stegemann, 1966; Desborough and Peloquin, 1969). Potato albumins were first fractionated into 3 fractions on a Sephadex G-100 column by Nakasone *et al.* (1972).

SDS-PAGE was used in a few reports of the molecular weight determination of the potato proteins (Stegemann, 1970; Stegemann *et al.*, 1973). Stegemann *et al.* (1973) have reported recently an extensive study on the molecular weight distribution of potato proteins. They have shown that proteins of dormant tubers, when treated with SDS (with no reducing agent) showed three major proteins of molecular weight, 16,800, 18,000 and 19,500, followed by a minor group of proteins at molecular weights, 34,500, 36,500 and 39,500, about a tenth in concentration. When a reducing agent (mercaptoethanol) was used in addition to SDS, the faster of the main subunits split into at least 2 even faster migrating zones at molecular weight 13,800 and 10,200. The size distribution of proteins in mature tubers was almost independent of the cultivar, indicating that differences in electrophoretic patterns of the different cultivars is mainly due to differences in the charge on the proteins. Part of the diversity in charge distribution was due to the different degrees of amidation. Treatment at pH 10 (causing deamidation) changed the cultivar dependent pattern to a result similar in all cultivars.

2.5. *Amino Acid Analysis*

2.5.1. Ion-Exchange Chromatography

The level of development and sophistication of amino acid analysis by ion-exchange chromatography (IEC) on automated systems is very high. Many modifications have appeared since the introduction of the first automated amino acid analyzer (Spackman *et al.*, 1968). They are all aimed at increasing sensitivity and reducing analysis time. Analysis of nanomole quantities is now routinely obtained in less than 90 minutes.

The order of elution of protein amino acids from a column of ion-exchange resin depends on the resin type, but in principle is as shown in the results obtained here (Figure 10) (for amino acid abbreviations see Appendix 1). Acidic amino acids elute first, while the basic ones emerge last from the column. The negatively charged resin repels molecules with negatively charged side chains, whereas it strongly binds basic amino acids whose side chains are positively charged at the low pH of the eluting buffers. Beside the charge effect, other forces contribute to the elution order. These are usually referred to as the hydrophobic interactions which affect the order of elution of aliphatic non-polar amino acids. Branching and hydroxylation accelerate elution (Hamilton, 1966). Detection and quantitation of the amino acids is based on the ninhydrin reaction (Blackburn, 1968).

Protein hydrolysis in 6N HCl (commonly used) causes quantitation problems. The amides (asparagine and glutamine) are hydrolysed to the corresponding amino acids (aspartic acid and glutamic acid). Serine and threonine are partially degraded (Downs and Pigman, 1969). Tryptophan is degraded to an extent that no reliable quantitation can be obtained. A collection of a few methods used for tryptophan determination was given by Friedman and Finley (1971). Different procedures have been reported for quantitative determination of cystine and cysteine, because of their degradation in acid hydrolysis (Blackburn, 1978a). The presence of excessive quantities of carbohydrates during acid hydrolysis has been reported to cause greater damage of amino acids, especially of tyrosine and methionine (Gordon and Basch, 1964). This is of importance in regard to non-purified potato proteins amino acid analysis. However, methionine and tyrosine degradation can be prevented by adding phenol to the hydrolytic system (Blackburn, 1978a). A limited degradation of other amino acids can occur under the routine hydrolytic conditions (6N HCl at 110°C for 24 hrs) (Roach and Gehrke, 1970). In the analysis of free amino acids, the omission of the hydrolysis step eliminates most of these problems.

2.5.2. Amides Determination

The amides (asparagine and glutamine) play an important role in nitrogen metabolism in plant (Miflin and Lea, 1977). The deamidation of these amides under experimental conditions is of concern from the analytical point of view (Robinson *et al.*, 1970). The amides are completely deamidated during hydrolysis of proteins in 6N HCl. Hence, they are measured together with the corresponding amino acids. The estimation of total amides in proteins can be obtained by following the course of slow deamidation under mild hydrolytic conditions. Correction for ammonia liberated from other amino acids is done by extrapolation to zero time (Leach, 1953).

Amide determination in a free amino acid extract bypasses the need for hydrolysis. But, unfortunately the amides are not well separated by ion-exchange chromatography in the classical procedure employing sodium citrate buffer as column eluant. They emerge from the column as one peak (Moore *et al.*, 1958). Therefore a sample has to be analyzed twice, hydrolysed and non-hydrolysed and the amides content determined by difference. A procedure for amino acid analysis of physiological fluids including the separation of amides using lithium citrate buffer system was developed by Benson *et al.* (1967) and Benson (1972). However, difficulty in the glutamine determination using a similar system was reported recently (Elmore and King, 1978). An interesting method, although lengthy, for amides determination in potato free amino extract was suggested by Talley *et al.* (1964). An aliquot of the potato extract was treated with phosphate buffer pH 6.7 for 90 min at 100°C, in order to cyclize glutamine to pyrrolidonecarboxylic acid. The treated extract containing the cyclized amide was applied to the ion-exchange column. The fraction corresponding to pyrrolidonecarboxylic acid was collected from the column effluent before reaction with ninhydrin. The fraction collected was hydrolysed in concentrated HCl, then the acid removed and the sample rechromatographed. The glutamine content of the original sample of extract was assumed to be equivalent to the pyrrolidonecarboxylic acid in the cyclized sample.

The amides instability and the analytical difficulties in their determination have resulted in considerable effort to solve these problems, but so far there is no one completely satisfactory method for their determination.

2.5.3. Amino Acid Composition of the Potato

An important characteristic of potato nitrogen distribution is the occurrence of sometimes more than 50% of the nitrogen in the non-protein fraction (NPN) form (Steward and Street, 1946; Li and Sayre, 1975). This is a problem because potato amino acids in the free amino acid pool contribute significantly to the NPN (Markakis, 1975).

The total amino acid composition of the potato was reported recently by a few investigators. The overall profile of amino acids showed high content of aspartic and glutamic acids and high essential amino acids content in comparison to other plant proteins (Kaldy and Markakis, 1972; Eppendorfer, 1978). In these works special precautions were taken in the determination of the sulfur containing amino acids and methionine was found the limiting amino acid of the potato. However, Rexen (1976) showed that in several cultivars, isoleucine was the limiting amino acid.

While the amino acid composition of potato proteins is relatively constant for a given cultivar (Schuphan, 1970), the composition of the free amino acid (FAA) pool is affected by climate, fertilizer, storage and chemical treatments (Mulder and Bakema, 1956; Hoff *et al.*, 1971; Vigue and Li, 1976). In most of these studies aqueous ethanol solutions of various concentrations were used for the extraction of the free amino acids (FAA) of the potato. Most commonly used are solutions of 70-80% ethanol. Recently, Kapoor *et al.* (1975b) have evaluated several solvents for the extraction of non-protein nitrogen (NPN). They found that dilute acids (trichloroacetic acid, sulphosalicylic acid and acetic acid) yielded a substantial quantity of protein nitrogen in addition to NPN in the extract from potato. Comparing these results with that obtained by 80% ethanol they concluded that the latter was the most suitable solvent for extraction of NPN, because little of the protein nitrogen was present in the extract. As well, about 75% of the NPN was contributed by FAA. Glutamic and aspartic acids and valine were the three major amino acids detected and constituted more than 50% of the FAA pool.

The potato FAA profile was reviewed by Synge (1977) and extensively studied by Davies (1977). The amides, aspartic and glutamic acids, valine and arginine appeared as the major constituents of this fraction. In addition to the common amino acids which are present in low amounts there were reports of the following non-common amino acids in the FAA pool of the potato; γ-aminobutyric acid (GABA), α-aminobutyric acid, β-alanine, ornithine, L-pipecolic acid and S-methylmethionine (Synge, 1977). Only GABA appeared in large quantity. The first reference to this substance as a constituent of the potato tuber was by Dent *et al.* (1947). Excluding the amides from the alcohol soluble FAA of the potato GABA appeared to comprise 11% of this fraction (Thompson *et al.*, 1953). It does not occur combined in proteins. Its role in nitrogen metabolism in plants was reviewed by Dixon and Fowden (1961).

2.5.4. Gas Chromatographic Analysis of Amino Acids

Gas-liquid chromatography (GLC) provides the analyst with some potential advantages for amino acid analysis: 1) High sensitivity, 2) High speed of analysis, 3) Diversity, 4) Lower initial costs and 5) The ease of interfacing the gas chromatograph (GC) with a mass spectrometer, which was applied for identification of amino acids by Leimer *et al.* (1977). In spite of these advantages GLC has failed to displace the amino acid analyzer (Coulter and Hann, 1971). However, the separation and estimation of amino acid enantiomers by GLC may well assume a dominant role (Leimer *et al.*, 1977).

The GLC is a most powerful tool for separation of volatilizable compounds. Amino acids are not volatile due to their zwitterionic structure. They are strongly polar compounds with a very low vapour pressure. Their conversion into volatile compounds is achieved by derivatization, through which the electrostatic charges on the molecule are blocked. A large number of amino acid derivatives as tested by GLC were reviewed by Blau (1967) and more recently by Hušek and Macek (1975). The N-acylalkyl ester derivatives seem to be the most widely used. The correct nomenclature for the acylated esters should be N(O,S)-acylalkyl esters since all of the reactive groups are acylated. However, the shortened form N-acylalkyl is commonly used (Hušek and Macek, 1975). The derivative developed in this work (N-heptafluorobutyryl-isopropyl ester) belongs also to this type of derivatives.

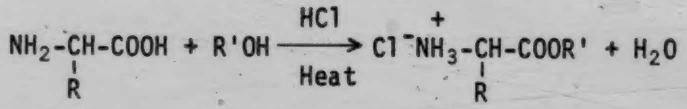
The fundamental work of Gehrke and co-workers (Gehrke *et al.*, 1968) was the basis for much of the subsequent developments in amino acid analysis by GLC. Their method was based on a two-step esterification procedure, followed by acylation with trifluoroacetic anhydride (TFAA). This was improved to one-step esterification by Roach and Gehrke (1969) and others. TFA esters of amino acids are sensitive to moisture and their low stability (Islam and Darbre, 1972) led to the introduction of heptafluorobutyric anhydride (HFBA) as the acylating agent. The use of HFBA to promote volatility of amino acids was first reported by Pollock (1967). It combines the desirable features of both TFAA and acetic anhydride. It forms more stable derivatives, which are less volatile than the corresponding N-TFA esters, so that the reaction medium (HFBA and ethyl acetate) can be evaporated with less loss. HFBA was used as the acylating reagent in a single column mode of operation in combination with a wide spectrum of alcohols as esterification agents of the carboxyl group of the amino acids (methanol, Kolb and Hoser, 1973; propanol, Moss *et al.*, 1971; butanol, Zumwalt, *et al.*, 1971; isobutanol, Mackenzie and Tenaschuk, 1974; isoamyl alcohol, Zanetta and Vincendon, 1973).

The general scheme of operations and reactions involved in derivatization of amino acids into their acylalkyl esters is given below:

Where R = side chain of amino acid;

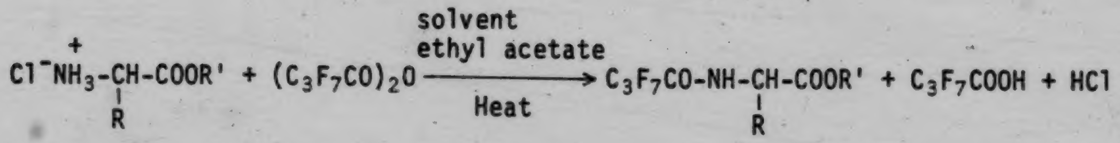
R' = alkyl group (CH_3, C_2H_5, C_3H_7, etc.)

1. Evaporation of water from sample.
2. Esterification with an alcohol to form an alkyl ester hydrochloride



$$NH_2\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}COOH + R'OH \xrightarrow[\text{Heat}]{HCl} Cl^-\overset{+}{N}H_3\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}COOR' + H_2O$$

3. Evaporation of the esterification reagents.
4. Acylation of alkyl ester hydrochlorides with an appropriate acylating agent, most widely used in recent years is, HFBA.



$$Cl^-\overset{+}{N}H_3\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}COOR' + (C_3F_7CO)_2O \xrightarrow[\text{Heat}]{\substack{\text{solvent}\\ \text{ethyl acetate}}} C_3F_7CO\text{-}NH\text{-}\underset{\displaystyle R}{\underset{|}{CH}}\text{-}COOR' + C_3F_7COOH + HCl$$

The separation and identification of the derivatized amino acids is most commonly carried out on a packed glass GLC column. The all-glass column system is preferred because the breakdown of N-trifluoroacetyl-*n*-butyl esters (TAB) occurred in a heated metal injection port (Lamkin and Gehrke, 1965; Stalling and Gehrke, 1966). A limited application of GLC analysis for the potato proteins and amino acids was by Hoff *et al.* (1971), who studied the effect of nitrogen fertilization on the composition of the free amino acid pool of the potato, using the TAB. The same derivative was also applied for amino acid analysis of potato protein bodies from the outer cortex of the tuber (Hoff *et al.*, 1972). The free amino acid pool of the potato was also analyzed by March (1975), using the N-HFB-*n*-propyl ester derivative.

The combination of a gas chromatograph and a mass spectrometer (GC-MS) for identification of amino acids in biological samples is being used more widely (Summons *et al.*, 1974; Abramson *et al.*, 1974). The mass spectra of the acylalkyl ester derivatives of amino acids have been studied by several investigators. The most comprehensive study by Leimer *et al.* (1977) presented complete mass spectra and characteristic fragmentation patterns for 48 TAB derivatives of amino acids.

2.6. *Processing Effects on Potato Free Amino Acids*

There has been a steady increase in annual output of processed potato products (Talburt, 1975). Dehydrated mashed potato granules are one of the major potato products in Alberta. They are produced by the Add-Back (A-B) process (Olson and Harrington, 1955), shown schematically in Figure 3. Recently a new process for the production of potato granules was

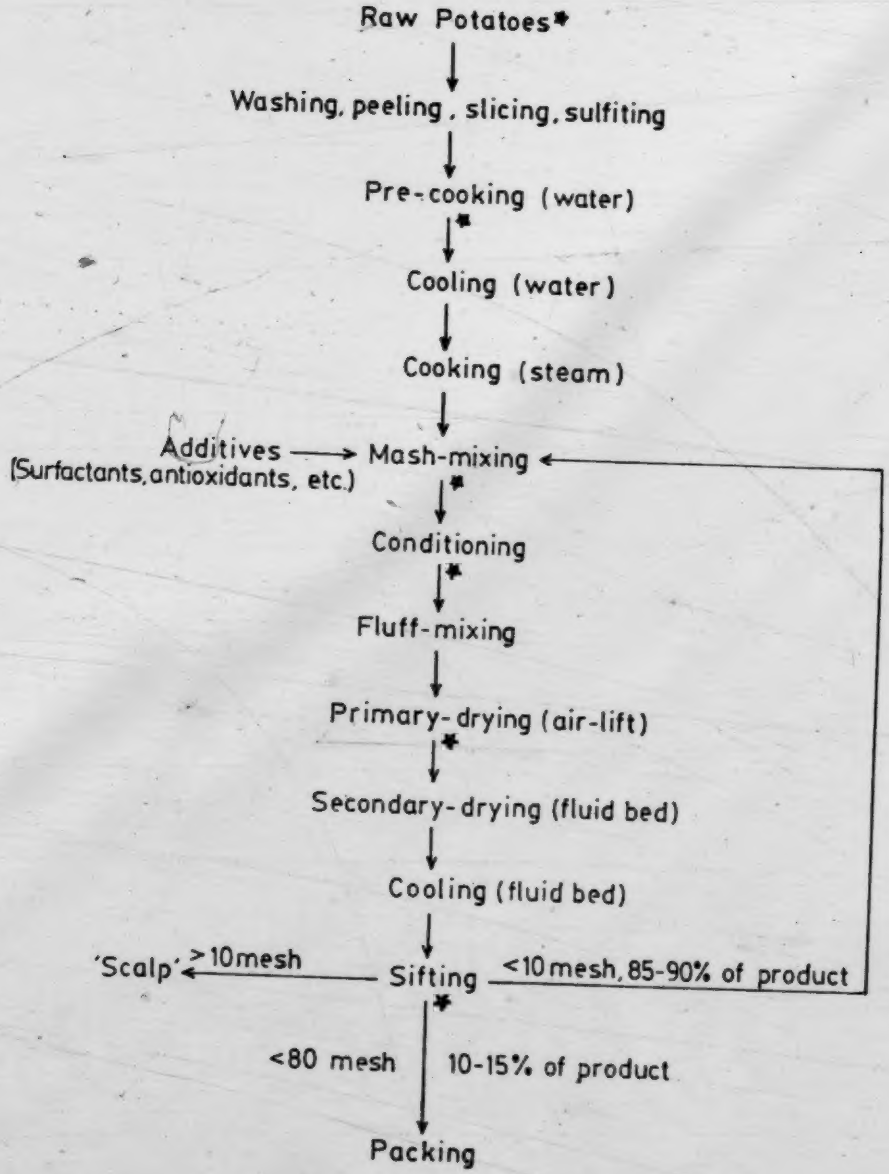

Raw Potatoes*
Washing, peeling, slicing, sulfiting
Pre-cooking (water)
*
Cooling (water)
Cooking (steam)
Additives
(Surfactants, antioxidants, etc.)
Mash-mixing
*
Conditioning
*
Fluff-mixing
Primary-drying (air-lift)
*
Secondary-drying (fluid bed)
Cooling (fluid bed)
'Scalp'
>10 mesh
Sifting
<10 mesh, 85-90% of product
*
<80 mesh
10-15% of product
Packing

Figure 3. Flow chart of the Add-Back process. (Asterisks[*] indicate the position in the process where samples were taken for the study reported in this thesis.)

developed by Ooraikul (1977).

Heat treatment is a most important step in home cooking as well as the industrial scale processing. It can be beneficial, as in the case of legume proteins, where anti-nutritional factors are destroyed and the nutritive value of the protein is increased (Liener, 1976). However, it also can be detrimental due to chemical reactions or mere physical leaching of amino acids and other solutes to the cooking medium. The major reactions involved in the loss of amino acids during processing were reviewed recently by Mauron (1972) and Cheftel (1977).

In view of the fact that a significant portion of potato amino acids are in the free amino acid state (Markakis, 1975), the effect of processing on this fraction can be of importance to processors. Information on amino acid composition of potatoes and the way it is affected during processing is scanty (Jaswal, 1973). Amino acid analyses of raw and cooked potato proteins and amino acid by ion-exchange chromatography (in its early stages of development) showed large variations between samples and considerable discrepancies between analyses done by different investigators (Hughes, 1958). This was ascribed to possible differences in the material analyzed, as well as differences in analytical techniques. In addition, Hughes (1958) reported no changes in amino acid composition of boiled potatoes in comparison with the raw potato. In contrast to this observation, Desborough and Weiser (1974) reported a 50% loss of total protein nitrogen after boiling for 30 min. In a recent report on changes in nutrient composition of potatoes during home preparation (boiling, oven baking and microwave cooking) Toma *et al.* (1978) revealed that cooking, regardless of the method used, did not affect the protein content of Russet Burbank tubers, except in one case, where tubers were subjected to microwave treatment. In that case there was a significant increase in the protein content. The authors refer to this result as unrealistic. It appears that additional studies on the changes in protein quantity and quality of potatoes during boiling are required. The effect of chipping on the nitrogenous compounds of potatoes was studied by Fitzpatrick *et al.* (1965) and Fitzpatrick and Porter (1966). They found that the greater the accumulation of reducing sugars in stored potatoes, the greater was the loss of FAA after chipping. The loss of amino acids and amides nitrogen was 52% and 85% in the chipping of unstored and stored potatoes, respectively. In one case, 88.4% of the FAA and 88.7% of the sugars could not be recovered from the chips, presumably as a result of the Maillard browning reaction. But even when reducing sugars were not abundant, considerable quantities of FAA were lost during chipping. High losses of bound and free amino acids were reported by Jaswal (1973). All processing methods used (canning, drum drying, french frying, and chipping) adversely affected the available lysine content, with chips and canned potatoes showing the maximum loss, followed by drum dried and french fried potatoes.

Although free amino acids are nutritionally equivalent to protein bound amino acids, they are more readily leached and lost during processing and food preparation. Therefore, more studies of the effects of processing on the FAA pool of the potato are necessary.

3. MATERIALS AND METHODS

3.1. *Potatoes*

3.1.1. Raw Potatoes

The potatoes used in this study were grown on the University of Alberta farm. Raw potato tuber cultivars: 'Norland', 'Snowchip', 'Alaska Red', 'Red Pontiac', 'Kennebec' and 'Netted Gem' were kindly supplied by Dr. Wm. T. Andrew, Department of Plant Science, The University of Alberta. Mature tubers of the different cultivars were harvested on October 28, 1976. Immature 'Netted Gems' (the major cultivar in Alberta) were harvested during stages of tuber development on the dates specified in Table 2 (Section 4.1.1.). All cultivars were planted as small whole seeds, between May 20th and 25th. Soil nutrient levels were raised before planting to 200 lbs/ac. N, 95 lbs/ac. P_2O_5, 400 lbs/ac. K_2O, using fertilizer. Row spacing was 36" and space between plants 12". The plot was hilled twice on July 9th and 13th.

Yields obtained are listed below:

Cultivar	Cwt/ac.
'Norland'	306
'Snowchip'	376
'Alaska Red'	474
'Red Pontiac'	368
'Kennebec'	434
'Netted Gem'	408

Small quantities of the 'Netted Gem' cultivar were obtained from the university farm at other times during this research.

3.1.2. Processed Potatoes

Effects of the various steps in the Add-Back (A-B) process for potato granules production on the nitrogen and free amino acids content of the potato ('Netted Gem') during processing were examined. Samples were taken from the processing line at Vauxhall Foods Ltd. (Vauxhall, Alberta, Canada). The stages in the process where samples were taken are marked with asterisks in Figure 3.

3.1.3. Raw Potato Composition

Mature potato tubers of the different cultivars (tubers of similar size of each cultivar were chosen) and tubers in varying states of maturity during growth of the 'Netted Gem' were hand-peeled (approximately a 3 mm thick layer including both the periderm and part of the cortex was removed, see Figure 1). The peeled portion was designated outer layer (OL) and the rest of the tuber inner layer (IL). Each portion (peel only of mature 'Netted Gem') was diced, quickly frozen and freeze-dried in a model FFD-42-WS Freeze-Dryer (The Virtis Co. Inc., Gardiner, N.Y.). The dried material was powdered with a waring blendor, passed through a 60 mesh size net and stored in brown bottles at 4°C for further analysis. Dry matter content was determined by oven drying at 70°C for 48 hrs in a vacuum oven.

The micro-Kjeldahl method was used for nitrogen determination (AOAC, 1965). Approximately 50 mg of freeze-dried powder was digested with concentrated sulfuric acid, sp. gr. 1.84, N-free (Fisher Scientific Company Ltd.) in the presence of a catalyst (potassium sulfate/mercuric oxide; 40:1 w/w). The final digest was clarified by the addition of a few drops of 30% hydrogen peroxide. Distillation was done in a micro-Kjeldahl distillation unit. The distillate was titrated with standardized HCl solution (0.02N).

Procedures for amino acid analysis of the freeze-dried powder are described in Section 3.5.1.

3.1.4. Potato Proteins Solubility

Freeze-dried powder of the OL and IL (see Figure 1) of the 'Netted Gem' tuber was used in these experiments. A 1g sample was suspended in 50 ml distilled water containing 500 ppm Na_2SO_3, and stirred at room temperature (~23°C) for 2 hrs. The mixture was transferred quantitatively to a cellulose dialysis tubing, 3.3 cm in diameter (Fisher Scientific Co. Ltd.) and dialyzed against glass distilled water at 4°C in a 4 litre beaker for at least 60 hrs with at least 6 changes of the dialyzate. Non-dialyzable nitrogen was determined at intervals during the dialysis period.

After dialysis the sample was centrifuged in a Beckman Model J-21B centrifuge at 14,000 x g for 10 min. The supernatant collected and the pellet was resuspended in glass distilled water, stirred for 1 hr and again centrifuged. Then the pellet was resuspended in 0.02 M phosphate buffer at pH 7.5 containing 5% sodium chloride, stirred for 2 hrs, and centrifuged as before. This step was repeated. The residual pellet was then successively extracted twice with each of the following solutions; 70% ethanol, 0.1 M acetic acid and 0.2% sodium hydroxide. The solubilized fractions (the supernatants from each extraction treatment) were collected and designated albumins, globulins, prolamines and acidic and basic glutelins, respectively. Quantitative determination of protein in these fractions was done according to a modification of Lowry's method (Bailey, 1967) (see 3.2.1.1.).

3.1.5. Proteins Preparation for Electrophoresis

3.1.5.1. Water Soluble and Insoluble Proteins

The extraction buffer suggested by Nakasone *et al.* (1972) was used to extract total potato proteins. Ten grams of freeze-dried powder of 'Netted Gem' tubers were extracted with 100 ml 0.05 M potassium phosphate buffer pH 7.3, containing 5% sodium chloride and 1.5% sodium sulfite, by homogenization in a Sorval Omni-mixer Homogenizer (Ivan Sorval Inc., Norwalk, Connecticut, U.S.A.), at 4°C for 2 min. The suspension was then centrifuged at 14,000 x g for 10 min at 4°C. This procedure was repeated twice. The combined supernatants were dialyzed for 60 hrs against glass distilled water at 4°C and then centrifuged at 14,000 x g for 20 min. The insoluble fraction was pelleted and the supernatant containing the water soluble fraction was decanted and brought to 90% saturation with ammonium sulfate and left to precipitate at 4°C, overnight, then centrifuged as before. The precipitated fraction was dissolved in water and dialyzed at 4°C until free of salt; both protein fractions were freeze-dried and stored dessicated at 4°C. Resolubilization of the water insoluble fraction in the extraction solution was encountered with difficulties as was also reported by Nakasone *et al.* (1972). Varying salt concentration did not affect the solubility significantly. Solubilization was 74% based on total amino acid recovery (Table 10). This is a common phenomenon in extracted plant proteins (Stegemann *et al.*, 1973). It was attributed to denaturation and aggregation during extraction steps. Complete solubilization was obtained by SDS, DTT treatment before electrophoresis. In view of these difficulties a fast separation procedure of potato proteins, without purification steps, was used as outlined below.

3.1.5.2. Sap Proteins Preparation

The method suggested by Stegemann *et al.* (1973) was followed. Fresh 'Netted Gem' tubers were frozen at -20°C for 24 hrs, then thawed to room temperature. Then only the 'skin' (see Figure 1) was slipped away from the tuber. For whole potato sap the whole tuber was sliced into small cubes, which were wrapped in cloth and pressed to the maximum pressure obtainable on the hand operated Carver hydraulic press, Model B (F.S. Carver Inc., Summit, N.J.). Sap from the outer layer of the tuber was obtained similarly, after peeling approximately 3 mm of the outer layer of the tuber. The rest of the tuber was used for sap expression from the inner layer.

The expressed juice was collected and immediately mixed with freshly prepared sulfite solution (5% sodium sulfite + 3.75% metabisulfite), in a ratio of 50:1, respectively, and centrifuged at 14,000 x g for 10 min at 4°C in a Beckman Model J-21B centrifuge. The clarified supernatant containing approximately 10 mg/ml proteins without further purification was used for thin layer isoelectric focusing (TLIF).

3.1.6. Free Amino Acids Extraction

Free amino acids (FAA) were extracted from potato ('Netted Gem') samples obtained from the processing line in Vauxhall Foods Ltd. Three samples of approximately 100g each were picked up at the following steps in the process (marked with asterisks on Figure 3). 1. Peeled raw potatoes, 2. after pre-cooking, 3. after mash-mixing, 4. after conditioning, 5. after air-lift drying, 6. final product, 7. 'scalp' (coarse particles used for livestock feed). The samples were chilled in an ice cooler and a portion was separated for FAA extraction. The remainder was freeze-dried (freeze-dryer type, see Section 3.1.3.) within 16 to 36 hours. Dry weight and nitrogen was determined as described in Section 3.1.3. For FAA extraction, the procedure of Jaswal (1973), with some modifications was used. A sample corresponding to 5g (dry weight basis) was macerated in a Sorval Omni-Mixer Homogenizer in 250 ml 70% ethanol (taking into account the estimated moisture content of the sample) for 2 min, and then transferred to a 500 ml Erlenmyer and shaken for 8 hrs on a Burrell Wrist-Action Shaker (Burrell Corp., Pittsburgh, PA) at room temperature. The mixture was then centrifuged at 10,000 x g for 10 min on a Beckman Model J-21 Centrifuge. The precipitate was discarded and the supernatant passed through a membrane filter (Millipore) with a pore size of 0.45μ. The clear filtrate was evaporated to dryness on a rotary evaporator (Buchi/Brinkman, Brinkman Instruments (Canada) Ltd.) under reduced pressure and intermittent nitrogen flushes. The residue was dissolved in 0.1N HCl, final volumes varied between 10 and 20 ml. Each sample was divided into 3 vials and kept frozen until used for amino acid analysis.

3.2. *Chemical Determination of Proteins and Amino Acids*

3.2.1. Protein Determination

In the gel chromatography separation, protein content in the eluted fractions was monitored by UV absorption at 280 nm. Either the Unicam SP 1800 Ultraviolet Spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, England) or the Gilford 252 Photometer (Gilford

Instrument Laboratories Inc., Obetlin, Ohio) were used for spectrophotometric measurements.

3.2.1.1. Protein Determination With Folin-Ciocalteau Reagent

A modification of Lowry's method (Lowry *et al.*, 1951) as suggested by Bailey (1967) was used to quantitate proteins. The reaction tube contained 1 ml sample, 5 ml of a reagent containing 2% Na_2CO_3 in 0.1 N NaOH and 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate in a 50:1 ratio, respectively. This reagent was mixed before addition to the sample. The reaction mixture was left at room temperature for 10 min, then 0.5 ml of IN Folin-Ciocalteau solution (Fisher Scientific Co. Ltd.) was added to the mixture with thorough mixing. The absorption of the reaction mixture was measured spectrophotometrically after 30 min at 750 nm. Bovine Serum Albumin (BSA) 99% pure, fatty acid poor, B grade (Calbiochem, San Diego, Calif., USA) was used to prepare the standard curve (Figure 4). The range of concentrations from 0 to 100 μg BSA per assay tube, gave linear results.

3.2.1.2. Protein Determination by the Biuret Reagent

For concentrated samples a Biuret method (Bailey, 1967) was used for protein determination. The linear range for this method was 1 to 6.5 mg protein per reaction tube.

The reaction mixture consisted of 1 ml sample and 4 ml Biuret reagent, which was made up of 1.5g copper sulfate ($CuSO_4.5H_2O$) and 6.0g sodium potassium tartarate ($NaKC_4H_4O_6.4H_2O$) dissolved in 500 ml of water. To this solution 300 ml of 10% sodium hydroxide was added with thorough mixing, then 2.0g of potassium iodide was added. The solution was made up to 2 litres and stored in a polyethylene bottle. After mixing the

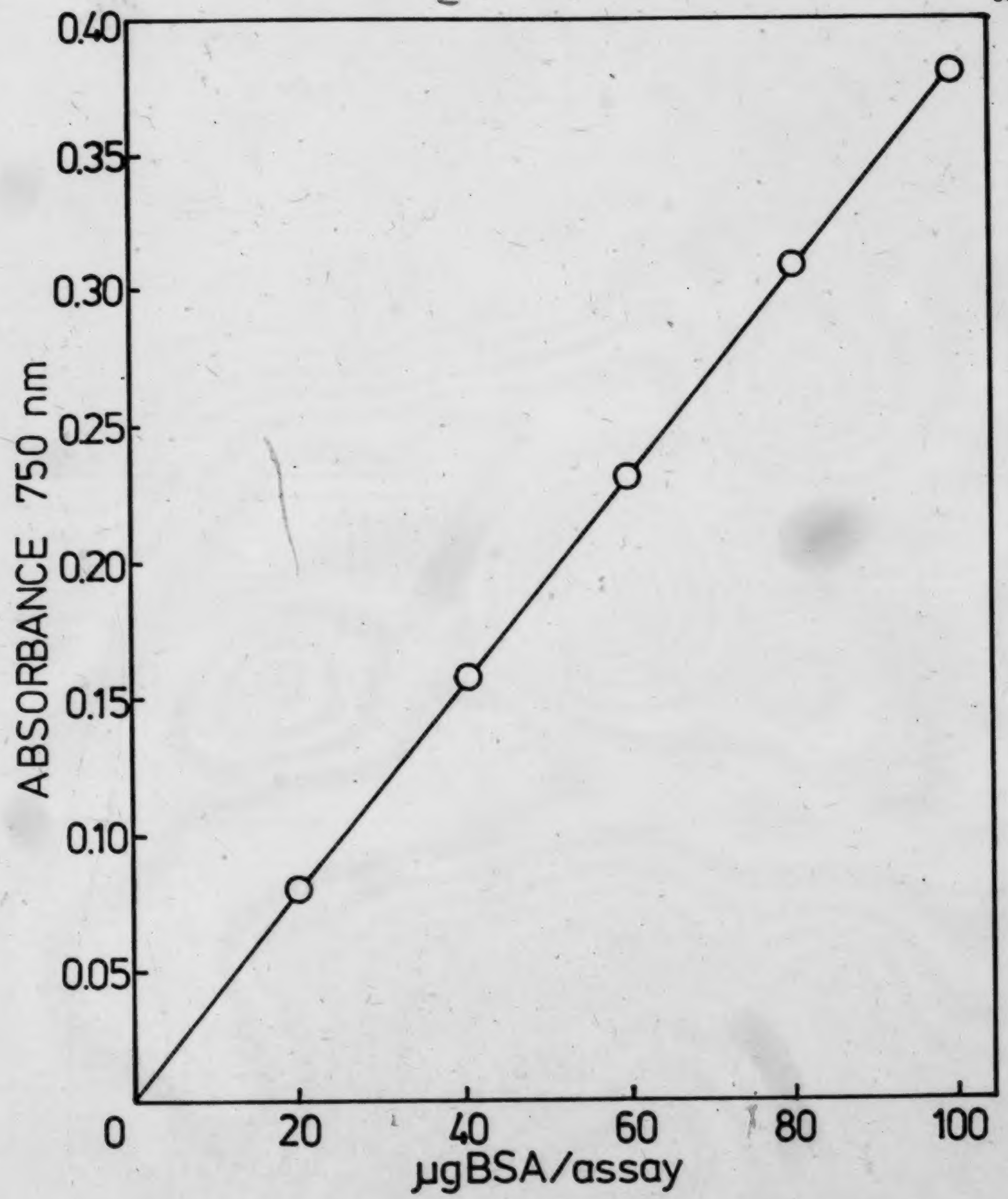


Figure 4. Standard curve for protein determination by Lowry's method.

sample with the reagent, the mixture was allowed to stand for 30 min, at room temperature. Absorption was measured at 540 nm; the standard curve was constructed using BSA as standard protein (Figure 5).

3.2.2. Free Amino Acids Determination By The Ninhydrin Method

The procedure proposed by Rosen (1957) was used with modifications, to determine free amino acids(FAA) content in potato extracts.

3.2.2.1. Reagents and Procedure

Solution 1: 0.01 M sodium cyanide (NaCN).

Solution 2: Acetate buffer pH 5.3-5.4, containing 540g sodium acetate ($NaOAc.3H_2O$), 400 ml H_2O, 100 ml glacial acetic acid and made up to 1500 ml with H_2O.

Solution 3: Acetate-cyanide solution, containing 20 ml of Solution 1 and made-up to 1 litre with Solution 2.

Solution 4: 3% ninhydrin (Cambrian Chemicals Co.) in methyl cellosolve (ethyleneglycol monomethyl ether; Fisher Scientific Co.), freshly prepared before addition to reaction mixture.

Solution 5: Isopropanol-water, 1:1 v/v (diluent).

The reaction mixture consisted of 1 ml sample, which contained 0.020-0.40 μmole of amino acid mixture, 0.5 ml of Solution 3 and 0.5 ml of Solution 4. It was heated for 15 min in a boiling water bath. Upon removal of the test tube from the water bath 5 ml of Solution 5 (diluent) was added and the tube well mixed. Absorption of the solution was measured at 570 nm, after 30 minutes of cooling at room temperature.

3.2.2.2. Potato Free Amino Acids

The major potato FAA, according to Synge (1977) and Davies (1977), were selected to construct the standard curve (asparagine, glutamine,

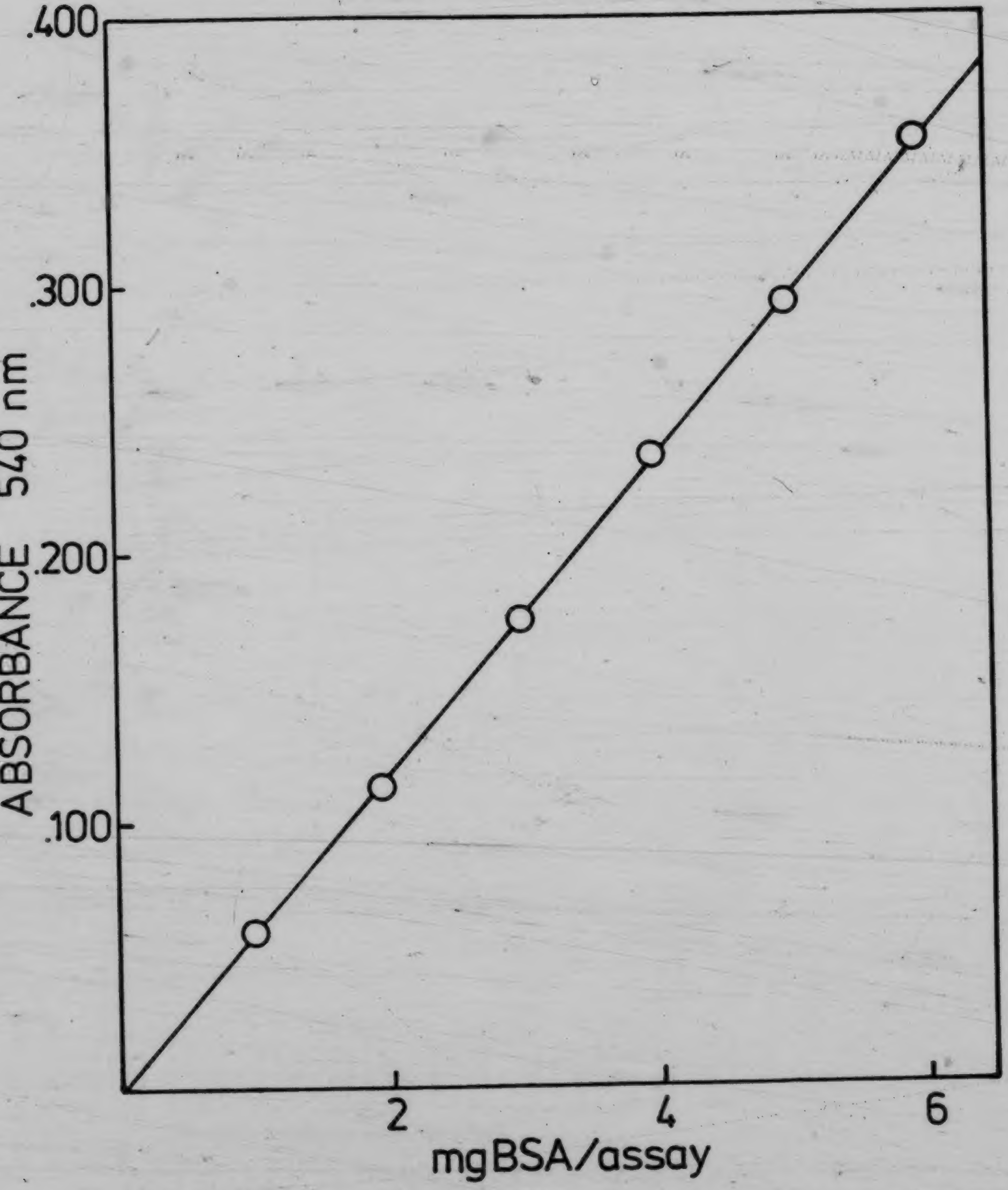


Figure 5. Standard curve for protein determination by Biuret method.

aspartic acid, glutamic acid, valine, leucine and arginine). Stock solutions of 0.4 μmole/ml of each standard amino acid and an equimolar mixture were prepared. The individual amino acids and the mixture were reacted with ninhydrin according to the procedure described in previous section (3.2.2.1.). The results from the equimolar mixture were used to construct the standard curve. The results of the individual amino acids were also linear, and virtually superimposable with the standard curve (Figure 6).

3.3. *Electrophoretic Techniques*

3.3.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Reagents and Procedure. Chemicals for PAGE were purchased from Eastman Kodak Company, unless otherwise specified. Acrylamide and sodium dodecyl sulfate (SDS) were recrystalized from chloroform and ethanol, respectively. Acrylamide, 27.72g, and Bis (N,N'-methylenebis-acrylamide) 0.616g were dissolved in 100 ml glass distilled water. In order to obtain 8.5 or 10% polyacrylamide gel, appropriate aliquots (9.0 or 10.58 ml, respectively) of the above solution were mixed with 3.0 ml of 500 mM sodium phosphate buffer pH 7.1, 1.5 ml of 2% SDS and 1.5 ml of 1% TEMED (N,N,N',N'-tetramethylethylenediamine) (J.T. Baker Chemical Co.). Total volume was made-up to 30 ml with 15 ml ammonium persulfate 0.14%. Gelation was done in glass tubes 10 cm long and 0.6 cm I.D. After polymerization, the tubes were placed in an electrophoretic cell (Buchler Instr., Fort Lee, N.J.). The electrode reservoirs contained 50 mM sodium phosphate buffer pH 7.1 and 0.1% SDS. Standard and potato proteins were treated similarly prior to application to the gel. Standard proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) are

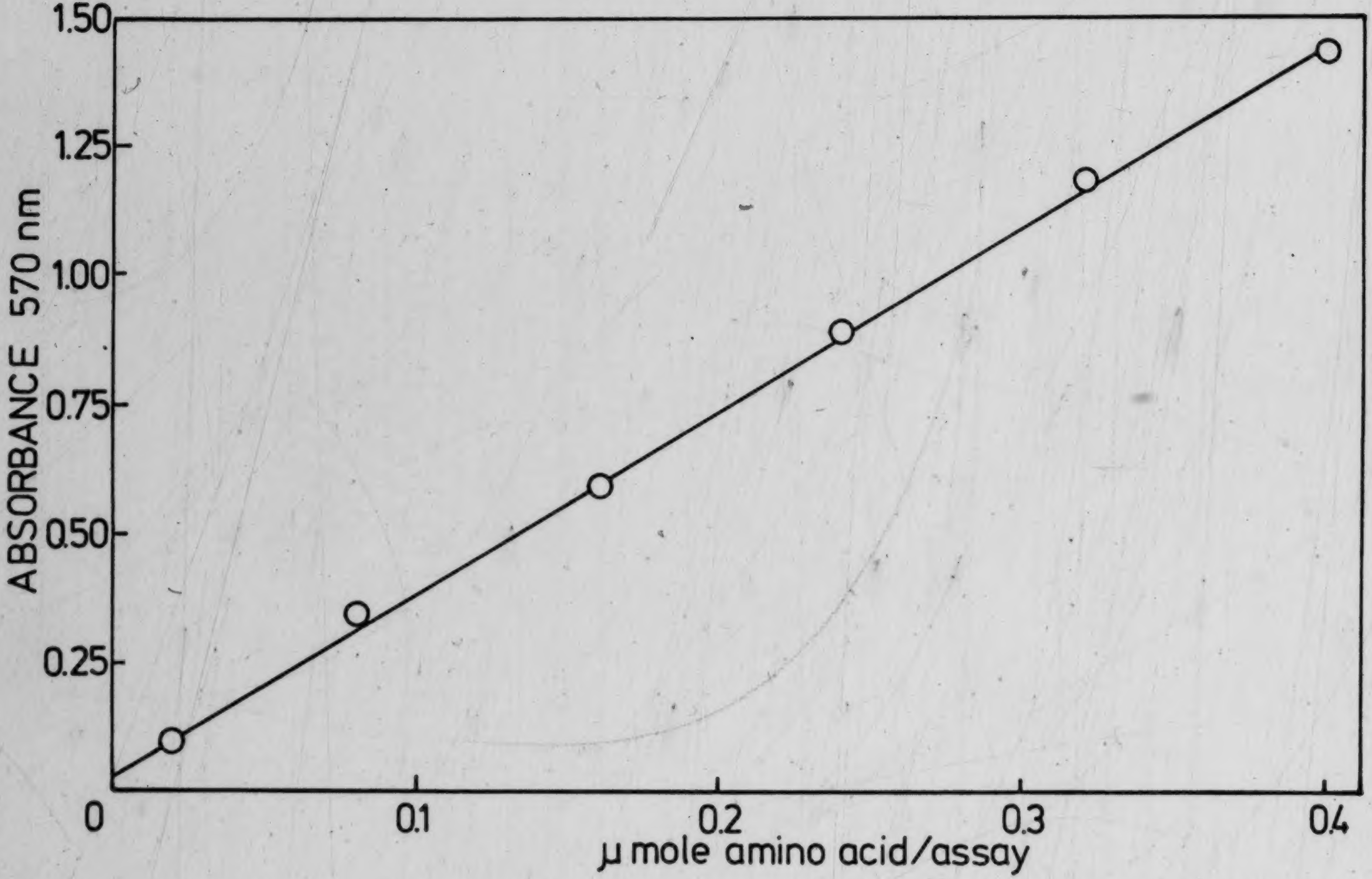


Figure 6. Standard curve for amino acid determination in a mixture by Ninhydrin method.

listed below:

Protein Standard	Molecular Weight
Aldolase	158,000
Ovalbumin	45,000
Chymotrysinogen A	25,000
Ribonuclease A	13,700
BSA (Calbiochem)	68,000

A protein solution of approximately 5 mg/ml was incubated for 20 min at 100°C in the presence of 1% SDS and 0.5 mM DTT (Dithiothreitol; "Cleland's reagent") (Calbiochem, LaJolla, Calif.). The solution was cooled to room temperature and mixed with 60% glycerol 1:1 ratio (v/v) to increase sample density. Protein samples in the range of 10-250 μg were applied to the gels. Electrophoresis was performed for 5 hrs at ~22°C at a constant current of 5 mA per tube. The gels were removed from the tubes under running water and stained overnight in 0.2% (w/v) Coomassie Brilliant Blue G-250 (Serva, Heidelberg) in a solvent mixture consisting of methanol, acetic acid, water (100:21:100). Excess stain was removed electrophoretically for 15 min, in a Canalco gel destainer (Canalco, Rockville, Md.) using an aqueous solution of 7.5% acetic acid and 5% methanol. The gels were photographed using an Asahi Pentax Spotmatic camera and Kodak Tri-X 400 ASA film. The negative film picture of the gels was then scanned on a Chromoscan Model MK II microdensitometer (Joyce Loebl, Gateshead, U.K.). Mobilities were calculated relative to chymotrypsinogen A, and the molecular weights of potato proteins were estimated by the standard curve (Figure 7) obtained from a plot of log molecular weight *vs* relative mobilities of the standard proteins.

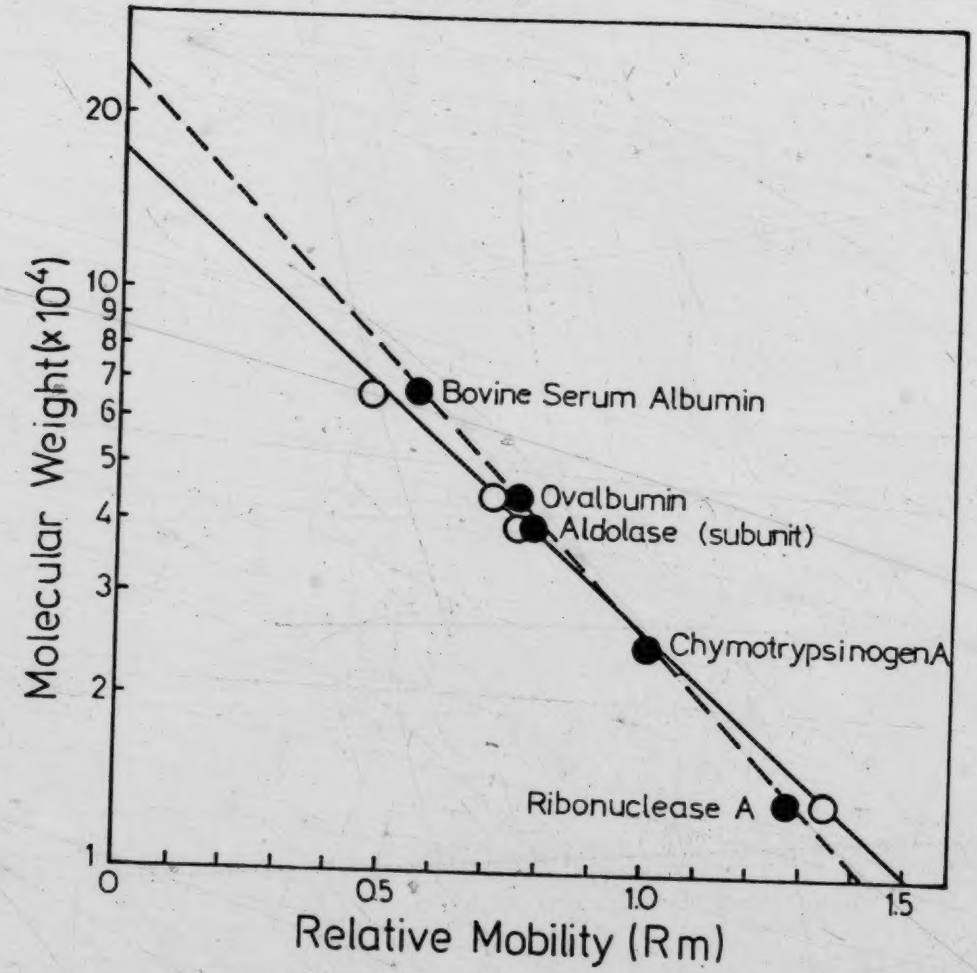


Figure 7. Standard curves for molecular weight determination. The equation for the line of best fit drawn through the data points is y = B x + A, where B = -0.98, A = 5.39 for 8.5% gel (---) and B = -0.81, A = 5.23 for 10% gel (-): y = log molecular weight and x is the distance of protein migration relative to chymotrypsinogen A.

3.3.2. Thin Layer Isoelectric Focusing (TLIF) in Granulated Gel

The methodology recommended by Radola (1973) was followed for TLIF. Glass plates (20 x 20 x 0.4 cm) were coated to approximately 1.0 mm thickness with a suspension of 7.5% (w/v) Sephadex G-75 superfine (Pharmacia Fine Chemicals) containing lysine and arginine at 0.05% each, and 1.5% (w/v) of ampholytes (Brinkmann, Westbury, N.Y.) in a pH range 2-10. The thin layer was obtained by pouring the gel mixture on the plate, spreading with a glass rod, then by tilting the plate, the suspension was spread evenly and smoothly on the surface of the plate. The plate was then allowed to reach the right consistency (loss of approximately 20-25% water) at room temperature for 30 min. Protein samples were usually applied as bands in the middle of the anodic region. The plate was placed on the cooling block of a TLIF double chamber (Desaga, Heidelberg, Germany). Filter wedge papers (2 mm thick) soaked in electrolyte bridge solution were layered on both edges of the plate and covered with Pt-ribbon electrodes. Aqueous solutions of sulfuric acid (0.2 M) and ethylenediamine (0.4 M) were used as anodic and cathodic electrolyte solutions, respectively. The cooling block was maintained at 5 ± 2°C using a low temperature water thermostat (Lo-temptrol M154, Precision Scientific, Chicago, Ill.). The electrophoretic run was performed at a potential of 200 V for 6 hrs, then increased to 800 V for 2 hrs.

Protein bands were detected by the paper print technique. A sheet of paper (140g/m^2, fast filtration type, No. 804, Macherey Nagel Co., Duren, Germany) was placed onto the gel layer and kept in contact with the gel for 2 min. The print was then dried in an oven at 110-120°C for 15 min after which the ampholytes were washed out with 10% (w/v) trichloroacetic acid by immersion of the print for 30 min in a small bath containing 100 ml of 0.2% (w/v), Coomassie Brilliant Blue G-250, in a solvent mixture of methanol, water and acetic acid 50:50:10 (v/v/v), respectively. The same solvents were used for destaining in a volume ratio of 33:66:10 (Radola, 1973). The paper print was photographed and the negative film scanned as described in Section 3.3.1. The pH gradient was measured at 25°C on approximately 2 x 20 mm streaks of the gel removed from the layer, at 1.0 cm intervals along the separation line. They were placed in the cavities of a white porcelain plate (Fisher Scientific Co. Ltd.), and liquified with approximately 100 μl glass distilled water. The pH was measured on a Metrohm pH meter Model E512 (Metrohm Ltd., 9100 Heresan, Switzerland), using a specially designed flat membrane glass electrode with a tip diameter of approximately 1.0 mm and equipped with a reference electrode (Desaga, Heidelberg, Germany).

3.4. *Column Chromatography of Water Soluble Proteins*

Column chromatography (gel filtration) was performed in a SR 25 glass column (100 x 2.5 I.D. cm). Sephadex G-150 40-120μ particle size was used as matrix for the separation of the proteins. The glass column and accessories, the standard proteins, Blue Dextran 2000 and the sephadex matrix were from Pharmacia Fine Chemicals (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Total column volume (Vt) was 357 ml and void volume (Vo) 122 ml, measured from elution volume of Blue Dextran 2000. The equation for calculation of the partition coefficient (Kav) was as given in Section 2.4.1. Packing of the column was done according to the Pharmacia Fine Chemicals instruction manual. Standard proteins listed in Section 3.3.1. were used to establish the calibration curve for molecular weight estimation (Figure 8). The extraction solution, 0.05 M potassium phosphate buffer pH 7.3 containing 5% NaCl and 1.5% Na_2SO_3, was used for column equilibration and elution. Approximately 100 mg of the freeze-dried water soluble proteins (extracted as described in Section 3.1.5.2.) were dissolved in 4 ml of the eluting buffer, and clarified by centrifugation at 1,900 x g for 10 min. The supernatant was applied to the column, eluted at a downward flow, maintained at 15.0 ± 0.5 ml/hr, 5.0 ml fractions were collected and monitored at 280 nm. Fractions corresponding to each peak were pooled together and protein determined by Lowry's method. The relative amount of the major peaks was determined, and their molecular weight estimated.

3.5. *Ion-Exchange Chromatography of Amino Acids*

3.5.1. Amino Acid Analysis of Protein Hydrolysates

Total amino acids analysis was done on hydrolysed protein samples. Approximately 2.0 mg freeze-dried potato powder or extracted protein samples were hydrolysed with constant boiling HCl (6N) containing 0.1% phenol at 105°C for 24 hrs, in an evacuated sealed glass tube. The hydrolysate was dried over sodium hydroxide pellets in a vacuum dessicator. The residue was dissolved in sodium citrate buffer pH 2.2 before application to the column. (Only in the comparison of the GLC and IEC methods for amino acid analysis were the hydrolysates dissolved in 0.1N HCl.)

Amino acid analysis of the hydrolysate was carried out on an automatic system, Durrum D-500 amino acid analyzer, in a single column

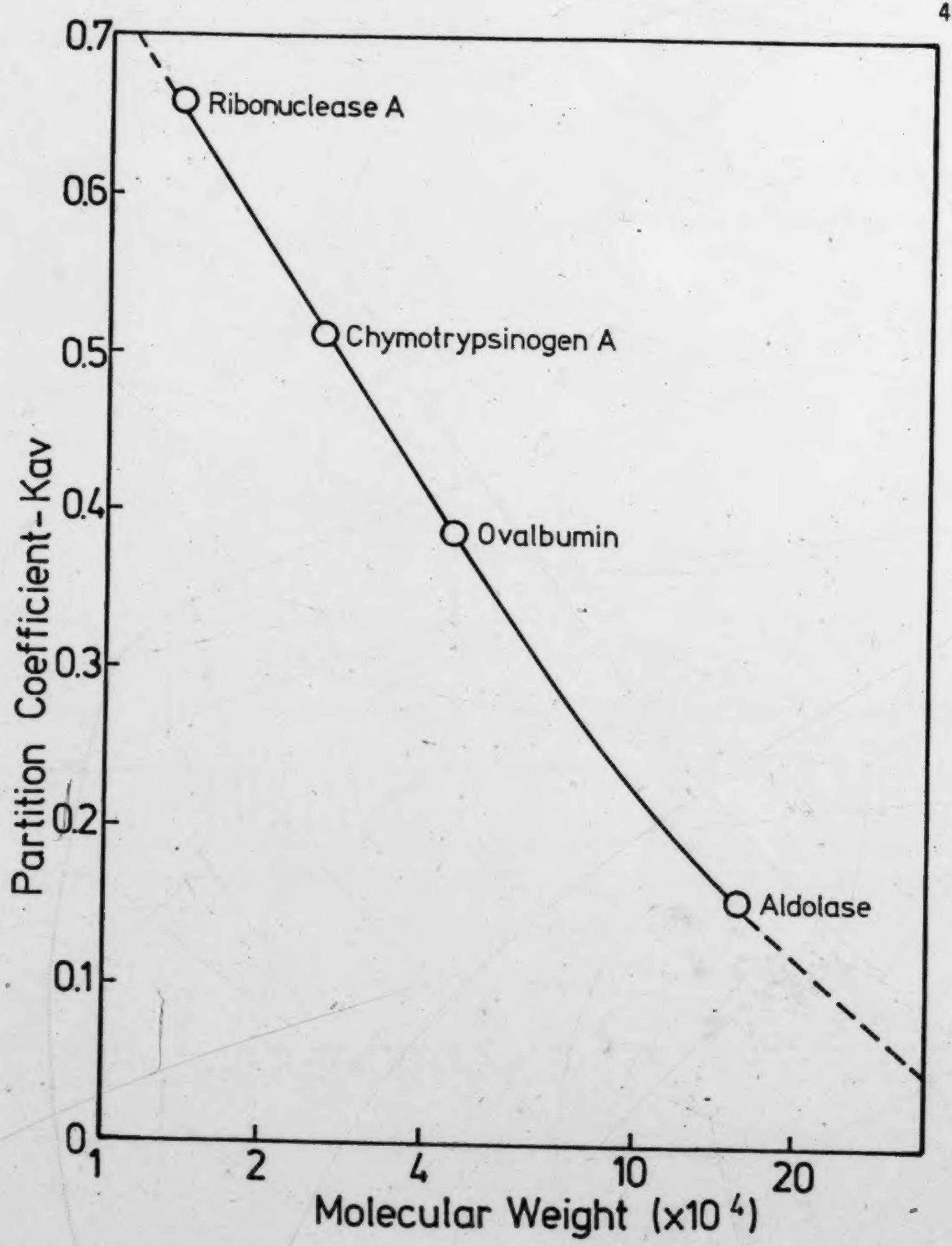


Figure 8. Standard curve for protein's molecular weight determination on column chromatography on Sephadex G-150.

operation (48 cm x 0.175 cm) using DC-6A cation-exchange resin. Analysis time per sample was approximately 82 min.

3.5.2. Asparagine and Glutamine (Amides) Analysis

3.5.2.1. The System for Analysis

The Beckman Model 120B amino acid analyzer equipped with the long column (56 x 0.9 cm, bed size), packed with type AA15 resin for acidic and neutral amino acids was used. Column temperature was maintained at 50°C. Elution buffer, 0.2N sodium citrate pH 3.28, containing 0.25% Thiodiglycol and 0.1% Phenol. The resin was regenerated every 4 hrs, by elution with sodium hydroxide 0.3N for 15 min, then re-equilibrated with the elution buffer before application of a new sample. Ninhydrin solution was prepared according to the Beckman instruction manual. Elution buffer and ninhydrin flow rates were maintained at 70 and 35 ml/h, respectively. The back pressure was 300 psi. Sample size applied to the column was constant, 0.5 ml.

3.5.2.2. Conditions for Amides Analysis

The standard amides (asparagine and glutamine) were determined as their corresponding amino acids after hydrolysis in concentrated (~12N) HCl (Fisher Scientific Co. Ltd.). The temperature (130° ± 5°C) and time (15 min) necessary for complete hydrolysis were determined emperically (see Section 4.2.1.). These conditions were used routinely for amides determination in the free amino acid pool of the potato. The AA15 column was used to separate the amides from the rest of the amino acids, and to determine the glutamic and aspartic acids. The overlapping fractions of asparagine and glutamine were collected from the column effluent during the elution of the sample. The column effluent was diverted before the ninhydrin reaction chamber, and 16.3 ml between retention time 43 to 57 min collected in a 25 ml volumetric flask. The volume was made-up to 25 ml mark with glass distilled water. An aliquot was taken for the analysis. It was first brought to dryness, then hydrolysed as described above. The resulting aspartic and glutamic acids were rechromatographed and their quantities represent the amount of asparagine and glutamine present in the original sample.

The effect of storage conditions on the stability of asparagine and glutamine standards (Pierce, Rockford, Ill., USA) was examined. Solutions of 0.1N HCl containing 0.10 μmole/ml of asparagine and glutamine separately and in a mixture, all containing 0.1 μmole/ml cysteic acid (Sigma Chemical Company, St. Louis, Mo., USA) as an internal standard, were subjected to room temperature (~23°C), refrigeration temperature (~1°C) and freezer temperature (~-16°C) for up to 35 days. During this period of time aliquots from any one condition were analyzed for the degree of degradation of the amides.

3.6. *Gas Chromatographic Procedure for Amino Acid Analysis*

3.6.1. Reagents

Standard amino acid mixtures and individual amino acids were obtained from Pierce (Rockford, Ill., USA), except γ-aminobutyric acid (GABA) which was obtained from Sigma Chemical Company. Heptafluorobutyric anhydride (HFBA) was from Eastman Kodak (Rochester, N.Y., USA). Solvents: isopropyl alcohol, ethyl acetate, dichloromethane and acetic anhydride were redistilled according to Perrin *et al.*, 1966, and kept under nitrogen atmosphere. Solvents were drawn out by syringe through the rubber cap and the drawn volume was replaced with nitrogen.

The esterification reagent was prepared in two ways: 1. Dry HCl gas (Matheson, Coleman and Bell) was bubbled into the dried isopropyl alcohol, after passage through a sulfuric acid tower. Normality of HCl was determined roughly by weight, diluted to the required normality (3.15N) and checked by titration. These solutions were stored in propylene bottles at -20°C. 2. The alternative method was slow pipetting of acetyl chloride (200 μl) into dried isopropanol (1.0 ml) at 0°C according to Felker and Bandurski (1975) and Pearce (1977).

3.6.2. Apparatus

3.6.2.1. General

A Varian 2100 gas chromatograph (GC) equipped with a flame ionization detector, coupled to a Varian model A-25 recorder, 1 mV full scale. Integration was done manually (unless otherwise specified). U-shaped glass columns, 3.5 m x 2 mm I.D. (thick wall) were cleaned in methanol, acetone and hexanes, then silanized and washed again with methanol and acetone, and dried with a stream of dry nitrogen. Columns were packed immediately after cleaning, using 3% SE-30 on 80-100 mesh Gas Chrom Q (Applied Science Labs, State College, Pa., USA).

The glass column was connected to the GC using a special glass-metal connector (Golan-Goldhirsh and Wolfe, 1979a) described below. Column conditioning was carried out for half an hour at 100°C, then nitrogen flow was shut down and temperature increased to 300°C and held for about 90 minutes. After baking, the temperature was reduced to 100°C and nitrogen flow resumed. The column was reheated and left overnight at 275°C with the nitrogen flow on. In regular use the oven temperature was maintained at 250°C overnight.

3.6.2.2. Glass-Column Connector for a GC

The column connector is constructed of a glass tube (a, Figure 9) sealed to a metal (Kovar) tube (b, Figure 9) (joined at the University of Alberta, Department of Chemistry and also can be purchased from the Cajon Company, Cleveland, Ohio, USA) approximately 3 cm long and 1/4" I.D. A narrow glass tube (c) is sealed to the internal side of the glass to metal transition, inside the Kovar tube. This forms a continuous glass tube. To the top of the internal glass tube, another glass tube (d, Figure 9) is connected, with a funnel-shaped end with a slot for the carrier gas, which fits up against the septum on the injector side (on the detector side only a straight tube is needed). The length and diameter of this piece is chosen according to the specifications of the design of the injector and detector ports. The rest of the chromatographic column is sealed to the bottom of this tube at g (Figure 9). Connection of the column to the GC is done by swagelok type fittings, which fit over the metal tube b, as is done with a metal column, using front-ferrule (e), back-ferrule (el) and nut (f). The overall view of the connector, connected to injection port (h) is shown in Figure 9.

3.6.3. Derivatization

Samples containing 50 to 500 nanomole amino acids were dried under a stream of dry nitrogen in 1 or 3 ml Reacti-vials (Pierce, Rockford, Ill., USA) held in an aluminum heating block at room temperature. The last water traces were removed azeotropically using dichloromethane. The dried residue was dissolved in 200 μl of the esterification reagent, sonicated in an ultrasonic cleaner (Cole-Parmer Instrument, Ill., USA)

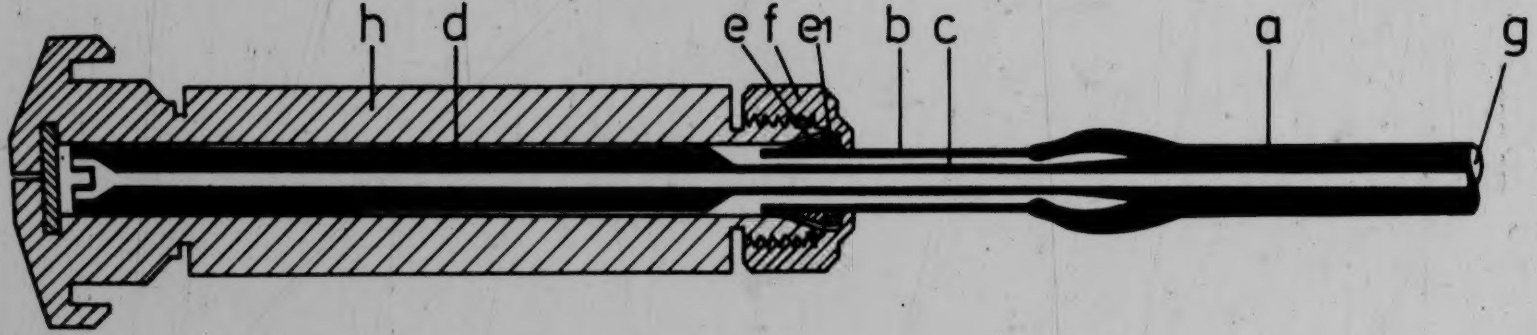


Figure 9. Glass column connector for a gas-chromatograph.

a = glass tube
b = metal (Kovar) tube
c = narrow glass tube
d = glass tube
e = front ferrule
e1= back ferrule
f = brass nut
g = connection to the rest of the column
h = injection port

for 1 min, and then placed in the heating block for esterification at 80°C for 120 min. After cooling the esterification solvent was evaporated. For acylation the residue was dissolved in 100 µl of ethyl acetate, 200 µl of HFBA added and the vial heated on the heating block for 10 min at 110°C. The vial was then cooled to room temperature, the reagent evaporated and the residue dissolved in an appropriate volume (25-100 µl) of ethyl acetate. Most of the derivatives were stable for a few weeks when kept in the freezer.

3.6.4. Chromatographic Conditions and Operations

The single column mode of operation was used. A flow rate of 30 ml/min was used for hydrogen and carrier gas (nitrogen). The air flow rate was 300 ml/min. The derivatized sample was mixed with acetic anhydride in a ratio of 1:1 in the syringe, in order to obtain a single peak for histidine, according to Moss *et al.* (1971), and then injected. 5 and 10 µl syringes, Hamilton 701-N (Hamilton Company Inc., Whittier, Ca., USA) were used. The oven temperature was held at 70°C for 1 min, then temperature programmed from 70°C to 225°C at a rate of 20°C/min (or as otherwise specified), and held at 225°C until complete elution of the sample was obtained. Injector and detector temperatures were maintained at 225°C and 280°C, respectively. The amount of each amino acid analyzed was in the range between 1 to 50 nmoles, which was within the linear range of detector response. Column performance under above operation conditions was evaluated by calculating efficiency and resolution. Efficiency in terms of theoretical plates per foot ($\bar{N}$) was calculated (>3000) for the proline peak. Resolution between the less resolved peaks of leucine and isoleucine was 0.86 and 0.79 for temperature programming at 8°C/min and 20°C/min, respectively (see Section 3.8).

3.6.5. Application to Protein Hydrolysates

Proteins bovine serum albumin (BSA) 99% pure, fatty acid poor, B grade (Calbiochem, San Diego, Calif., USA) and acid precipitated caseins (kindly provided by Dr. P. Jelen, Dept. of Food Science, University of Alberta) were hydrolysed in 2 ml hydrochloric acid at 105°C for 24 hours, in an evacuated sealed glass tube. The hydrolysate was dried over sodium hydroxide pellets in a vacuum dessicator. The residue was dissolved in 2 ml 0.1N HCl and analyzed by: 1. ion-exchange chromatography (see Section 3.5.1.), 2. the gas chromatographic method developed here, using conditions of 120 minutes esterification at 80°C, by the alternative method, followed by 10 minutes acylation at 110°C.

3.6.6. Clean-Up Procedure of FAA Extract for GC

Prior to derivatization of a potato FAA extract, it was passed through a small column of a cation-exchange resin, Rexyn 101 (H), exchange capacity 5.5 meq/g (Fisher Scientific Co. Ltd.). The column was made by pushing a small quantity of glass wool well down into the stem of a pasteur pipet (Dispo pipets; ASTM Designation; E 438-71, General Glass, McGraw Park, Ill., USA). Approximately 110 mg of the resin was then washed with glass distilled water into the column. The resin settled down fast, and its surface was left covered with water until sample application. This procedure was adapted from Adams *et al.* (1977).

Sample preparation for the clean-up involved mixing 100 µl of thawed FAA extract diluted 10 times with H_2O + 200 µl of a solution of 25% acetic acid in order to bring the pH of the sample between 2.0 and 2.5. 'Scalp' samples were also analyzed after addition of 10 µl ornithine (4.4 µg) as an internal standard. The mixture was transferred to the moist resin using a pasteur pipet, allowing to pass through the column at about one drop per 5 sec. Sample tube was washed with 200 µl glass distilled water and transferred to the column, followed immediately by 0.5 ml distilled water and the flow rate was slightly increased using a rubber bulb attached to the top of the column. The amino acids were eluted by passing through the column 1.0 ml of 2N NH_4OH at about one drop per second. The eluate was collected in a reacti-vial, in which the derivatization was carried out after evaporating the solvent.

3.7. *Integrated Gas Chromatograph-Mass Spectrometer (GC-MS) System*

The GC-MS combination was used for the identification of γ-aminobutyric acid and ornithine in the potato FAA pool.

The GC-MS data were obtained on a Varian 1400 GC, oven coupled via a porous ceramic Watson-Bieman helium separator to an AEI MS12 mass spectrometer. A coiled glass column identical in every other respect to the U-shaped column used for routine analysis of amino acid derivatives was employed. Helium flow rate was 30 ml/min and the temperature program was: isothermal at 70°C for 1 min, then programmed from 70 to 225°C at 8°C/min, then isothermal at 225°C for up to 25 min. The injector was maintained at 225°C and the inter-connecting lines, separator and ion source at >225°C.

Data acquisition and processing was by means of an AEI DS-50S data system with spectra acquired every 7 secs.

3.8. *Statistical Analysis and Calculations*

Statistical analysis of data was done on the University of Alberta computer, AMDHAL VI, using standard APL statistical package.

The GLC column performance was evaluated by calculating the efficiency and resolution. Efficiency is usually expressed in plates per foot. It was calculated from the following equations.

1. The number of theoretical plates, N.

$$N = 16 \left(\frac{T_R}{W}\right)^2,$$

where, T_R and W are in the same units

T_R = retention time (or distance)

W = width of the peak at the baseline

2. Plates per foot, $\bar{N}$.

$$\bar{N} = \frac{N}{L}$$

where, L = column length in feet.

Resolution (R) was calculated by the following equation:

$$R = 2 \left(\frac{T_{R_2} - T_{R_1}}{W_2 + W_1}\right)$$

where: T_{R_1} and T_{R_2} are the retention times of two adjacent peaks (1 and 2).

W_1 and W_2 are the widths at the baseline of peaks 1 and 2.

A resolution of R = 1 is generally considered as complete separation.

Relative molar response (RMR) of amino acids for the GLC method was calculated according to the following equation, where ornithine was used as the internal standard.

$$\mathrm{RMR}_{aa/is} = \frac{\text{amino acid molar response}}{\text{ornithine molar response}}$$

aa = amino acid

is = internal standard

RMR of ornithine was assigned a value of unity.

Relative mole % was calculated according to the following equation:

$$\text{Relative mole \% for an amino acid} = \frac{\dfrac{A_{aa}}{\mathrm{RMR}_{aa/is}}}{\Sigma\,\dfrac{A_{aa}}{\mathrm{RMR}_{aa/is}}} \times 100$$

A_{aa} = area of amino acid peak on chromatogram.

The calculation of μmole amino acid per gram dry weight in 'scalp' FAA extract using the internal standard (Table 17) was as follows. The amount of each amino acid present in the sample injected to the column was calculated by the following equation:

$$g_{aa} = \frac{A_{aa} \times MW_{aa} \times g_{is}}{A_{is} \times MW_{is} \times \mathrm{RMR}_{aa/is}}$$

where: g_{aa} = gram amino acid; g_{is} = gram internal standard; MW_{is} = molecular weight of internal standard

and:

$$\mu\text{mole aa/g dry weight} = \frac{\dfrac{g_{aa}}{MW_{aa}} \times \text{dilution factor}}{\text{g dry weight of original sample}}$$

Other common analytical lab procedures and data calculations were used as required.

4. RESULTS

4.1. *Raw Potato Protein Composition*

4.1.1. Proteins and Amino Acids in Different Cultivars and During Tuber Development

The nitrogen content of the potato is well documented in the literature (Talburt *et al.*, 1975). A wide range of values was reported according to cultivar and conditions of growth. In this work Alberta grown potatoes were evaluated for tuber size, peel portion, dry matter and nitrogen content (Tables 1,2). Differences were found among cultivars at maturity. The major cultivar grown in Alberta, 'Netted Gem', had a large tuber size and high dry matter content, while nitrogen content (1.5%) was relatively low. A similar relationship was found for 'Snowchip', which had high dry matter (25.4%) and low nitrogen content (1.5%). An inverse relationship was found in 'Norland' with low dry matter (17.8%) and high nitrogen content (2.5%). 'Alaska Red', 'Red Pontiac' and 'Kennebec' showed intermediate dry matter (22.6%) and nitrogen content (1.8-1.9%). When percent nitrogen is expressed on fresh weight basis, there were no differences among cultivars in total nitrogen content.

The changes in tuber size, peel portion, dry matter and nitrogen content were followed during growth of the 'Netted Gem' cultivar (Table 2). A continuous increase in dry matter content was obtained. The major changes occurred between July and August (15.8 to 17.3%) and September and October (20.6 to 25.7%).

The changes in nitrogen content did not show a clear trend when compared on dry weight basis. However, an increase in total nitrogen

Table 1. Potato tuber composition of different cultivars in terms of size, outer layer portion, dry matter and nitrogen content.

Cultivar*	Average fresh weight of tuber (g)	% outer layer (peel) of fresh weight	% dry matter	% nitrogen** of dry weight	% nitrogen of fresh weight
'Norland'	234.5	11.4	17.8	2.5	.4
'Snowchip'	218.9	10.1	25.4	1.5	.4
'Alaska Red'	192.1	10.1	22.6	1.9	.4
'Red Pontiac'	410.9	10.5	21.6	1.8	.4
'Kennebec'	240.1	9.1	23.6	1.9	.4
'Netted Gem'	367.4	10.0	25.7	1.5	.4

*Harvested in 1976/77 season. 10-15 tubers of each cultivar with average % CV = 15 for fresh weight were used.

**Average of duplicate determinations.

Table 2. 'Netted Gem' tuber composition during development in terms of size, outer layer portion, dry matter and nitrogen content.

Harvest* date	Fresh weight of tuber (g)	% outer layer (peel) of fresh weight	% dry matter	% nitrogen** of dry matter	% nitrogen of fresh weight
July 27	29.2	18.9	15.8	1.7	.3
August 16	57.2	20.9	17.3	1.4	.2
August 31	117.4	22.9	18.4	1.8	.3
September 9	123.3	17.8	19.2	1.7	.3
September 24	201.1	14.3	20.6	1.7	.3
October 28	367.4	10.0	25.7	1.4	.4

*Harvested in 1976/77 season. 10-15 tubers at each date were used. Average % CV = 20 for fresh weight.

**Average of duplicate determinations.

as percent of fresh weight was obtained, indicating accumulation of nitrogenous compounds in the tuber during growth. Manual peeling of the tuber (Table 1) resulted in a 10% loss on a fresh weight basis. The loss was higher (~20%) when the tuber was smaller (Table 2).

Amino acid composition of different cultivars as well as during 'Netted Gem' tuber development were determined using ion-exchange chromatography (Tables 3 and 4). A comparison of amino acid composition in different potato cultivars is shown in Table 3. A similar pattern of amino acids distribution was found in the cultivars tested. Aspartic and glutamic acids appeared to be the major amino acids in all cultivars. Their values represent the sum of the acid and corresponding amide, which was hydrolysed upon treatment with 6N HCl. γ-aminobutyric acid (GABA) was present in all the samples analyzed. It appeared on the chromatogram as a well resolved peak before tyrosine (Figure 10). Cystine was not detected. However, it is known to occur only in low amounts in the potato (Markakis, 1975). No special precautions were taken to prevent its degradation. Methionine was present in all cultivars in low amounts.

Amino acid composition of the 'Netted Gem' cultivar during growth is presented in Table 4. The overall trend observed was a decrease in the amount of all amino acids except aspartic acid and GABA. The lower total amino acid content at the last harvest is another expression of this trend (Table 4). Methionine was the only sulfur containing amino acid detected, as was the case in other cultivars. The 'essential amino acids' measured (threonine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine) were expressed

Table 3. Total amino acid composition[†] of potato cultivars.

Amino acid \ Cultivar	'Norland'	'Snowchip'	'Alaska Red'	'Red Pontiac'	'Kennebec'
			mg/g dry weight		
Asp*	22.04	22.33	12.44	18.39	17.44
Thr	3.73	3.11	2.90	2.60	3.38
Ser	3.42	2.93	2.96	2.50	2.98
Glu*	24.27	17.90	27.87	14.76	26.21
Pro	1.70	2.26	1.97	1.83	2.94
Gly	2.62	2.68	2.31	2.18	2.60
Ala	3.06	2.24	2.73	1.91	2.48
Val	4.50	4.10	4.20	3.35	4.52
Met	1.41	+	1.73	+	1.64
Ile	3.64	3.38	3.28	2.78	3.32
Leu	4.73	4.88	4.40	4.04	5.06
Tyr	2.89	2.95	3.15	2.43	2.73
Phe	3.90	3.65	3.90	3.04	3.88
GABA	3.05	1.66	1.81	1.64	1.97
His	1.74	1.81	1.50	1.56	1.65
Lys	5.36	4.70	4.72	4.36	4.98
Arg	4.43	3.84	4.85	3.26	5.79
Total	96.49	84.42	86.72	70.63	93.57
% EAA**	37.6	38.4	39.9	38.8	39.5

[†] Results are the average of at least three determinations. Average % CV = 6.

*Value represents the acid and the corresponding amide.

**$\% \text{ EAA} = \frac{\Sigma \text{ essential amino acids}}{\text{total amino acids}} \times 100$

+ Small peak detected.

Table 4. Total amino acid composition of 'Netted Gem' potatoes during tuber development.[†]

Amino acid \ Harvest date	Jul 27	Aug 16	Aug 31	Sept 9	Sept 24	Oct 28
			mg/g dry weight			
Asp*	14.58	14.08	17.87	17.83	18.50	20.15
Thr	4.19	3.73	3.40	3.28	2.97	2.74
Ser	3.60	3.27	3.21	3.05	2.96	2.73
Glu*	22.58	25.55	25.18	25.04	23.13	22.18
Pro	2.84	2.57	2.30	2.29	1.78	2.28
Gly	2.74	2.55	2.39	2.29	2.18	2.08
Ala	3.67	4.06	3.17	3.20	2.56	2.36
Val	7.13	5.94	5.16	4.93	4.25	3.68
Met	1.80	1.81	1.68	1.69	2.01	1.50
Ile	3.51	3.15	3.07	3.00	2.70	2.73
Leu	5.05	4.67	4.49	4.47	3.89	3.85
Tyr	2.96	2.72	2.68	2.82	2.42	2.80
Phe	4.82	3.75	3.54	3.70	3.08	3.25
GABA	1.06	1.60	1.41	1.68	1.73	2.57
His	1.41	1.17	1.38	1.29	1.07	1.13
Lys	4.76	4.15	4.30	4.20	3.82	3.88
Arg	4.98	4.32	4.16	4.21	3.88	4.05
Total	94.68	89.09	89.39	88.97	82.93	83.96
% EAA**	42.9	39.7	37.9	37.7	36.3	35.3

[†] Results are the average of three determinations. Average % CV = 6.

*Value represents the acid and the corresponding amide.

$$^{**}\%\ \text{EAA} = \frac{\Sigma\ \text{essential amino acids}}{\text{total amino acids}} \times 100$$

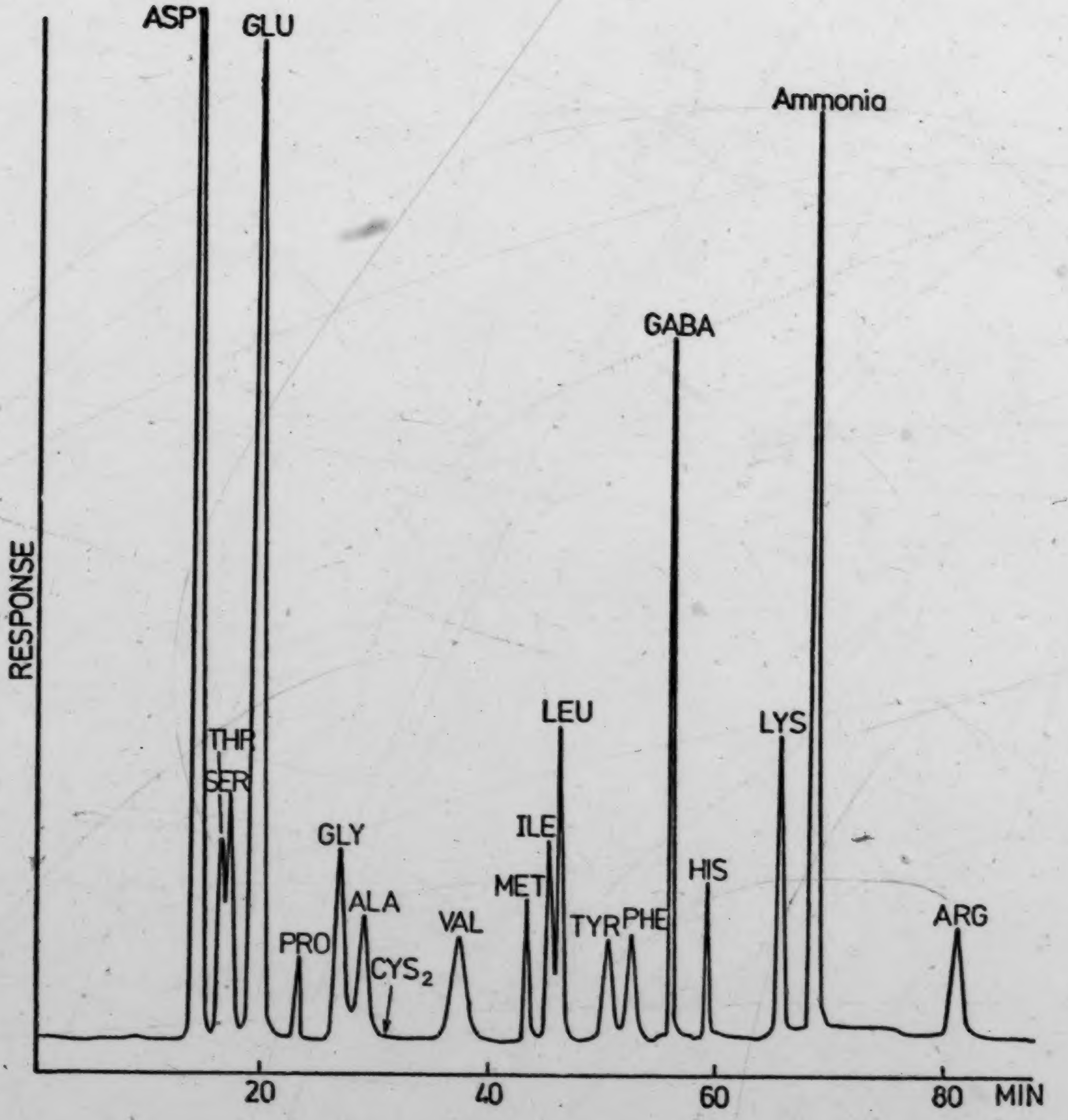


Figure 10. Chromatogram of amino acid analysis of hydrolyzed freeze-dried potato powder obtained on Durrum-500 amino acid analyzer, representing 0.2 mg dry weight of potato.

as percent of the total amino acids recovered and presented as % EAA (Tables 3 and 4). A similar approach for nutritional evaluation of potato amino acid pattern was used by Jaswal (1973). Similar % EAA were calculated for the different cultivars at maturity (Table 3). An interesting decrease in % EAA was found during 'Netted Gem' tuber growth, reaching the lowest value at maturity, 35.3% (Table 4).

Amino acid composition of potato in outer and inner layers of the tuber ('Netted Gem') is shown in Table 5. A characteristic chromatogram of amino acid analysis of the inner layer of the tuber, obtained by ion-exchange chromatography is demonstrated in Figure 10. The aspartic and glutamic acid peaks include the hydrolysed amides, asparagine and glutamine, respectively. The large ammonia peak was due to the ammonia released from the amides and to partial degradation of other amino acids, mainly serine and threonine. There was much higher content of total amino acids in the OL (150.11 mg/g) in comparison to the IL (83.96 mg/g) (Table 5). All amino acids in the OL, except glutamic acid and GABA appeared in a larger quantity than in the IL. The most significant amino acid in the OL was aspartic acid (50.24 mg/g). It was almost 2.5 times the glutamic acid (19.11 mg/g). In the IL these two acids contributed similar amounts, 20.15 and 22.18 mg/g, respectively. % EAA in the OL was higher than in the IL (Table 5) and similar to the values calculated for the other cultivars (Table 3). All essential amino acids were in larger amounts in the OL compared with the IL. Especially higher were leucine, histidine and lysine: 9.57, 2.56 and 8.46 mg/g, respectively.

Table 5. Total amino acid composition of outer and inner layers of the potato tuber ('Netted Gem').

Amino acid \ Part of tuber	Outer layer	Inner layer
	mg/g dry weight	
Asp*	50.24	20.15
Thr	5.73	2.74
Ser	5.64	2.73
Glu*	19.11	22.18
Pro	5.17	2.28
Gly	4.98	2.08
Ala	5.10	2.36
Val	7.15	3.68
Met	2.08	1.50
Ile	5.52	2.73
Leu	9.57	3.85
Tyr	5.19	2.80
Phe	6.54	3.25
GABA	2.31	2.57
His	2.56	1.13
Lys	8.46	3.88
Arg	4.76	4.05
Total	150.11	83.96
% EAA**	38.3	35.3

Results are the average of three determinations, average % CV = 6.

*Value represents the acid and the corresponding amide.

$$**\%\ EAA = \frac{\Sigma \text{ essential amino acids}}{\text{total amino acids}} \times 100$$

4.1.2. Solubility Pattern of Proteins of the Outer and Inner Layers of the Tuber

In order to characterize potato proteins according to solubility, freeze-dried potato powder ('Netted Gem') from the outer layer (OL) of the tuber and inner layer (IL), separately were subjected to prolonged dialysis against glass distilled water. Nitrogen content of the samples was measured at intervals during dialysis (Figure 11). There were no changes in nitrogen content beyond 60 hours of dialysis. The OL of the tuber contained significantly higher percent nitrogen (2.2 ± 0.2) compared with the IL (1.6 ± 0.1). This nitrogen distribution between OL and IL was also indicated by total amino acid composition (Table 5). The OL is usually lost in home cooking or industrial processing, prior to consumption of the potato. Percent non-dialyzable nitrogen (protein) in the OL was higher (1.0 ± 0.1) than in the IL (0.6 ± 0.1) (Figure 11). This fraction was 45.4% and 37.5% of total potato nitrogen in OL and IL, respectively.

The protein nitrogen fraction was fractionated according to solubility in different solvents. The potato protein fractions are shown in Table 6, according to solubility. Albumins comprised approximately 50% of total proteins recovered. The globulins comprised approximately one third of the proteins, 27.6% and 36.0% in OL and IL, respectively, and basic glutelins approximately 16%. A small amount of prolamines and acidic glutelins was found. Protein recovery was almost complete (96.2%) in the OL while in the IL recovery was complete (103.7%). Analysis of variance of the total proteins recovered by extraction and measured before extraction showed no significant

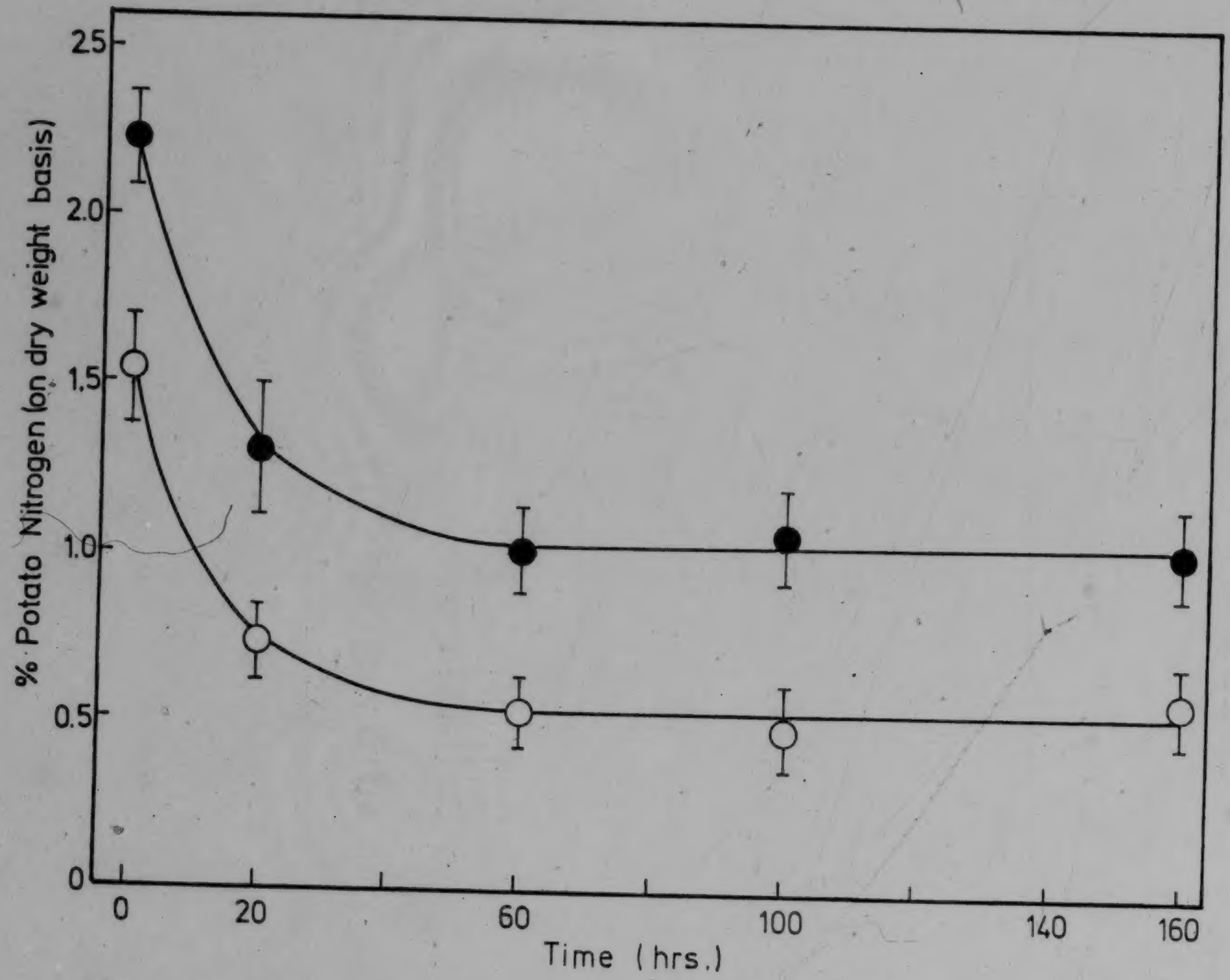


Figure 11. Changes in total potato nitrogen during dialysis of potato powder of inner (○–○) and outer (●–●) layers of the tuber.

Table 6. Potato protein fractions according to solubility, expressed on dry weight basis.

Solvent	Protein class	Outer layer mg/g	Outer layer % recovery	Inner layer mg/g	Inner layer % recovery
Glass distilled water	Albumins	49.94 ± 3.11	53.1	20.88 ± 1.80	46.4
5% NaCl in Phosphate buffer pH 7.5	Globulins	26.00 ± 1.60	27.6	16.21 ± 4.18	36.0
70% Ethanol*	Prolamines	2.05 ± 0.59	2.2	0.79 ± 0.23	1.7
0.1M Acetic acid	Glutelins	0.40 ± 0.05	0.4	0.16 ± 0.06	0.4
0.2% NaOH	Glutelins	15.69 ± 1.72	16.7	6.96 ± 1.61	15.5
Total recovered		94.08 ± 3.58	100	45.00 ± 4.57	100
Total measured before fractionation		97.82 ± 3.18		43.39 ± 2.86	

Protein determination was done by Lowry's method

*Ethanol had to be evaporated before protein determination.

difference at 1% level.

The solubility study showed that more than 50% of potato nitrogen is in a non-protein nitrogen (NPN) form (Figure 11). The free amino acid pool in the NPN was further studied in relation to the effect of the add-back process on potato nitrogen content (see Section 4.3). Potato ('Netted Gem') proteins were mainly composed of water soluble (albumins) and water insoluble (globulins) fractions. Therefore, further characterization of the water soluble and water insoluble proteins of the potato was attempted.

4.1.3. Electrophoretic Patterns of Potato Proteins

4.1.3.1. Polyacrylamide Gel Electrophoresis - PAGE

Potato extracted water soluble and water insoluble proteins were subjected to electrophoretic separation on 8.5% and 10% polyacrylamide gels. A schematic pattern of separation is shown in Figure 12. The combination of SDS,DTT treatment resulted in solubilization and separation of the complex protein molecules into smaller subunits of low molecular weight (Figure 12). Molecular weight of subunits obtained after SDS,DTT treatment were estimated by a standard curve (Figure 7). It was constructed by analyzing proteins of known molecular weight at the same conditions as were used for potato samples. A linear relationship existed between the log of molecular weight and relative mobility. In the extrapolated regions of the curve at the high and low molecular weight ranges, deviation from this line may be high (Weber and Osborn, 1969; Dunker and Rueckert, 1969). The standard curve obtained on 8.5% gels showed a steeper slope than the one obtained on 10% gels (Figure 7). This effect of gel concentration on the slope of the

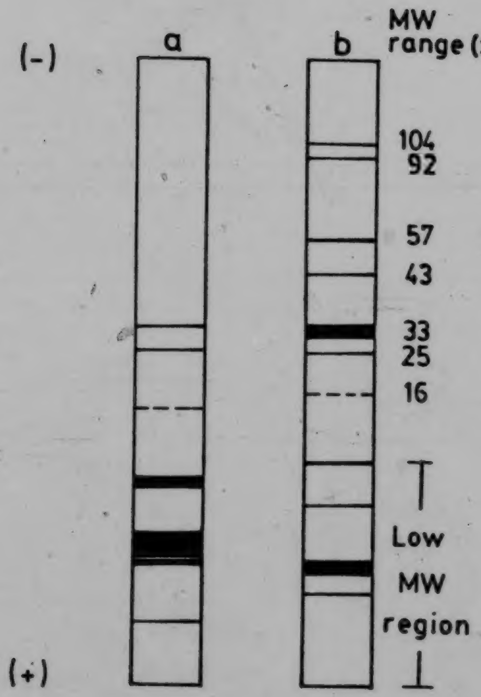

(-)
a
b
MW
range (x10^4)
104
92
57
43
33
25
16
Low
MW
region
(+)

Figure 12. Schematic electrophoretic pattern of separation of SDS,DTT treated potato proteins, and molecular weight estimates. Water insoluble (a), and water soluble (b) proteins.

standard curve was demonstrated by Dunker and Rueckert (1969). The effect of these gel concentrations on the separation of potato proteins is shown in Figures 13 and 14 water soluble and insoluble fractions, respectively. Scans of separation on 8.5% gel (a, Figures 13 and 14) and 10% gel (b, Figures 13 and 14) are shown. Similar patterns of separation were obtained on both gel concentrations. The major difference was in the faster migration of proteins on lower gel concentration (8.5%). Major protein bands appeared at the low molecular weight region, 10,000 and 30,000 (a,b, Figure 12).

4.1.3.2. Thin Layer Isoelectric Focusing on Granulated Gel-TLIF

Isoelectric focusing provided information about the potato proteins which was not obtainable by the method discussed earlier. TLIF on granulated gel in pH 2-10 ampholytes on a 20 cm separation distance enabled a determination of the isoelectric pH of the major proteins extracted from the potato. Electrophoregrams and scans of their negative film picture of water soluble, water insoluble fractions and sap proteins of potato ('Netted Gem') are shown in Figures 15, 16 and 17, respectively. The major protein bands in the water soluble fraction had isoelectric points (IP) at pH's 7.7, 7.0, 5.1, 5.0 and 4.7 (bands 3, 5, 9, 10, 12, Figure 15). In the water insoluble proteins only 8 bands were separated with the dominant ones at pH 8.5 region (bands 1, 2, Figure 16). These protein bands appeared in residual amounts in the water soluble fraction. On the other hand, band 7 in the water insoluble fraction (Figure 16) may be a residue from band 5 of the water soluble fraction (Figure 15). This indicated that the separation by solvent extraction and dialysis was

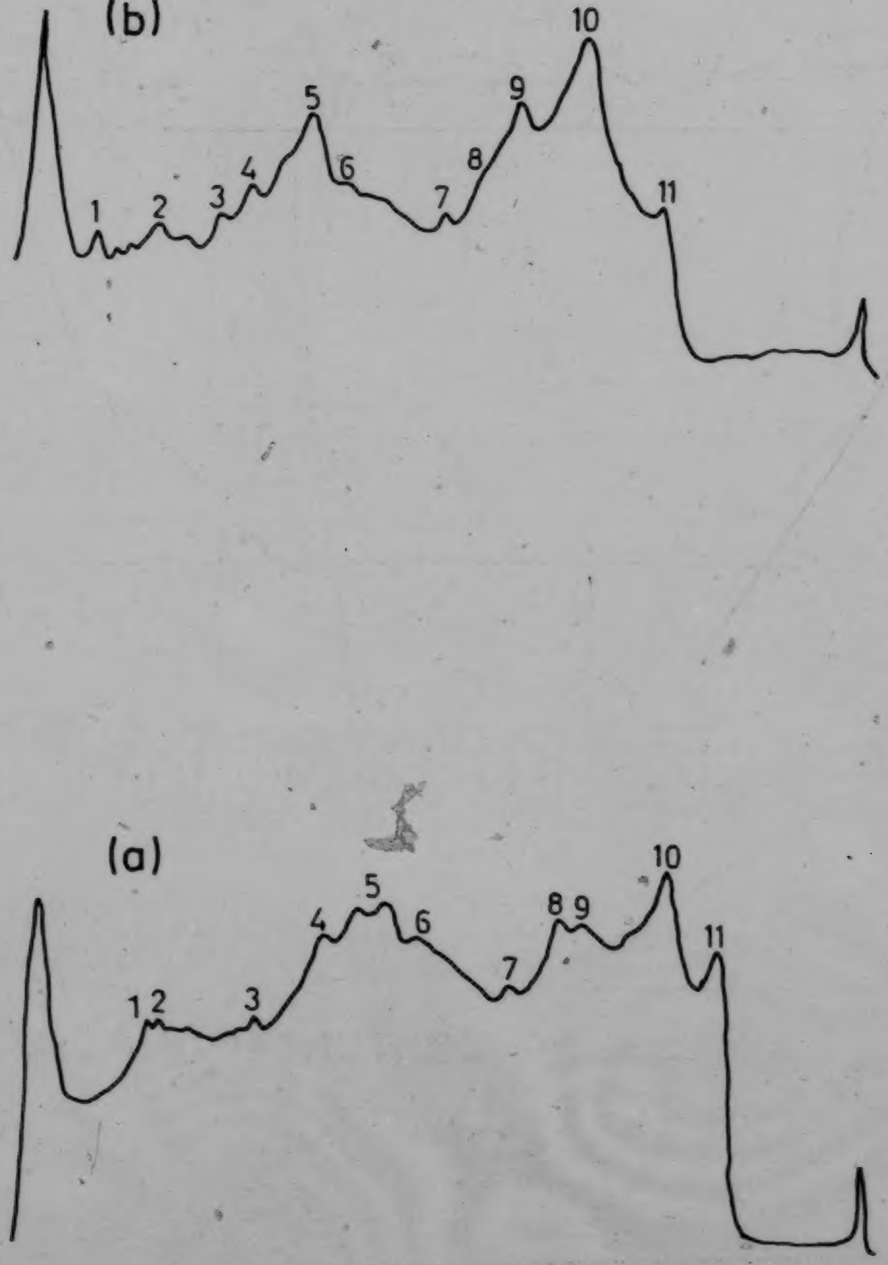


Figure 13. Scans of negative film picture of potato water soluble proteins, SDS,DTT treated on 8.5% (a) and 10% (b) polyacrylamide gel. 209 μg protein was applied to the gel.

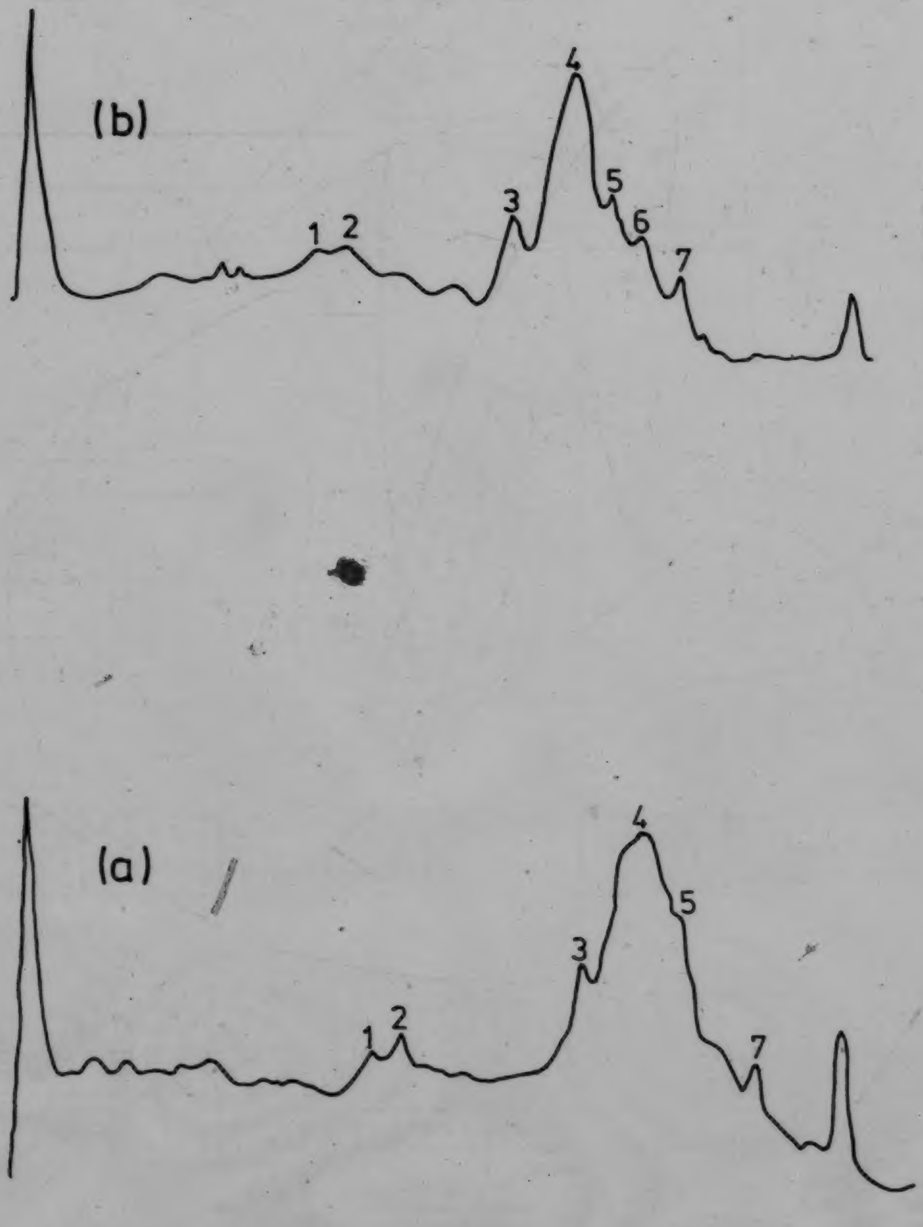


Figure 14. Scans of negative film picture of potato water insoluble proteins, SDS,DTT treated on 8.5% (a) and 10% (b) polyacrylamide gel. 147 μg and 88 μg protein was applied to the gels, respectively.

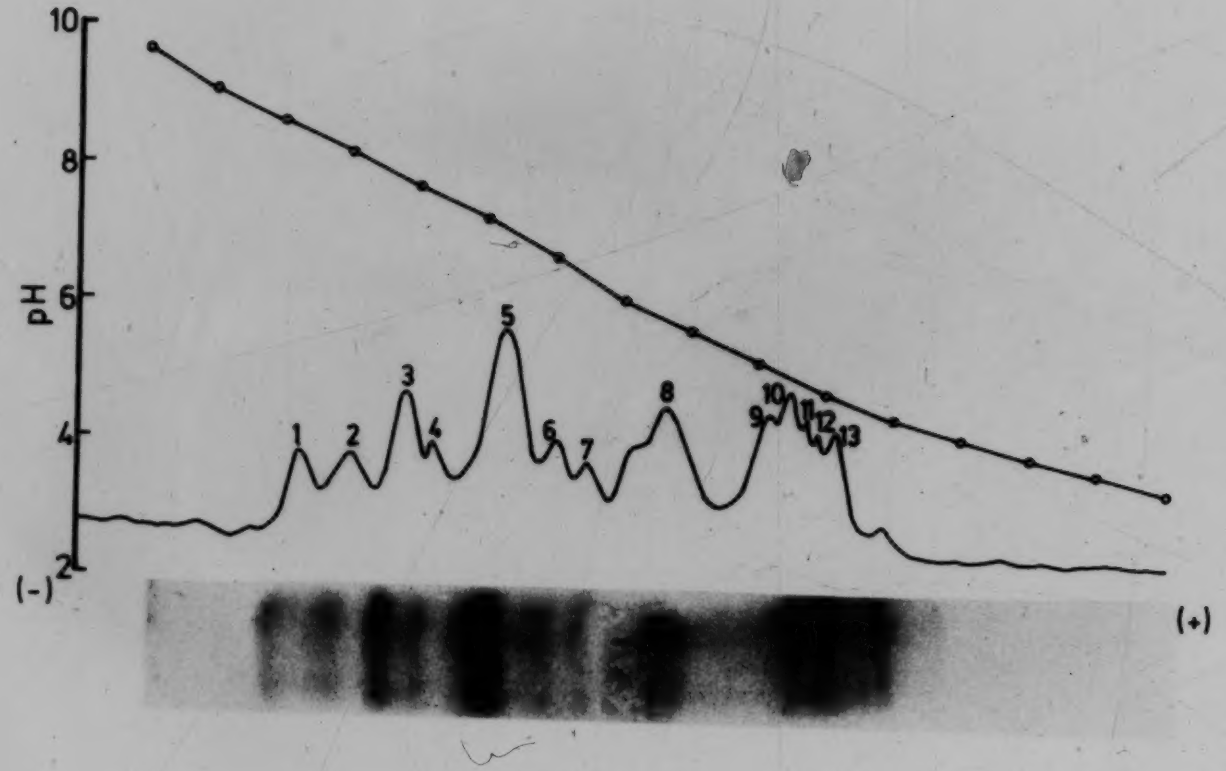


Figure 15. Scan of negative film picture of electrophoregram of potato water soluble proteins on TLIF, in pH 2-10 ampholytes. 459 μg protein was applied to the gel. pH gradient (o—o).

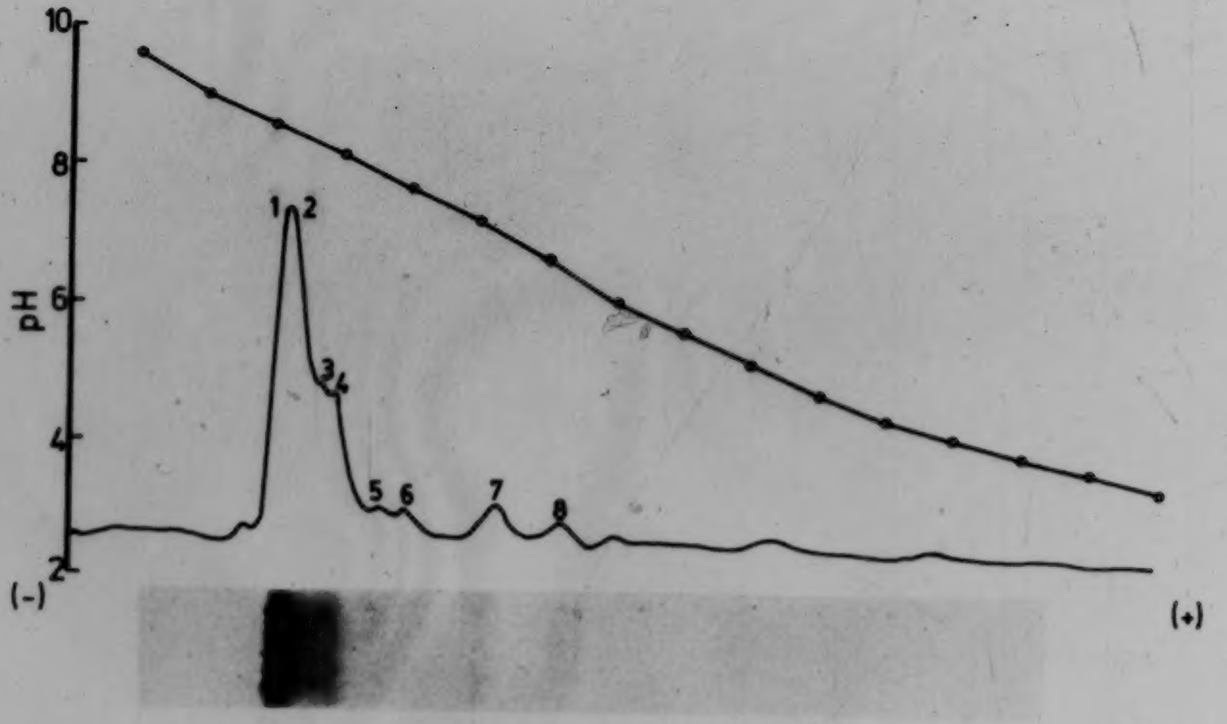


Figure 16. Scan of negative film picture of electrophoregram of potato water insoluble proteins on TLIF, in pH 2-10 ampholytes. 260 µg protein was applied to the gel. pH gradient (o—o).

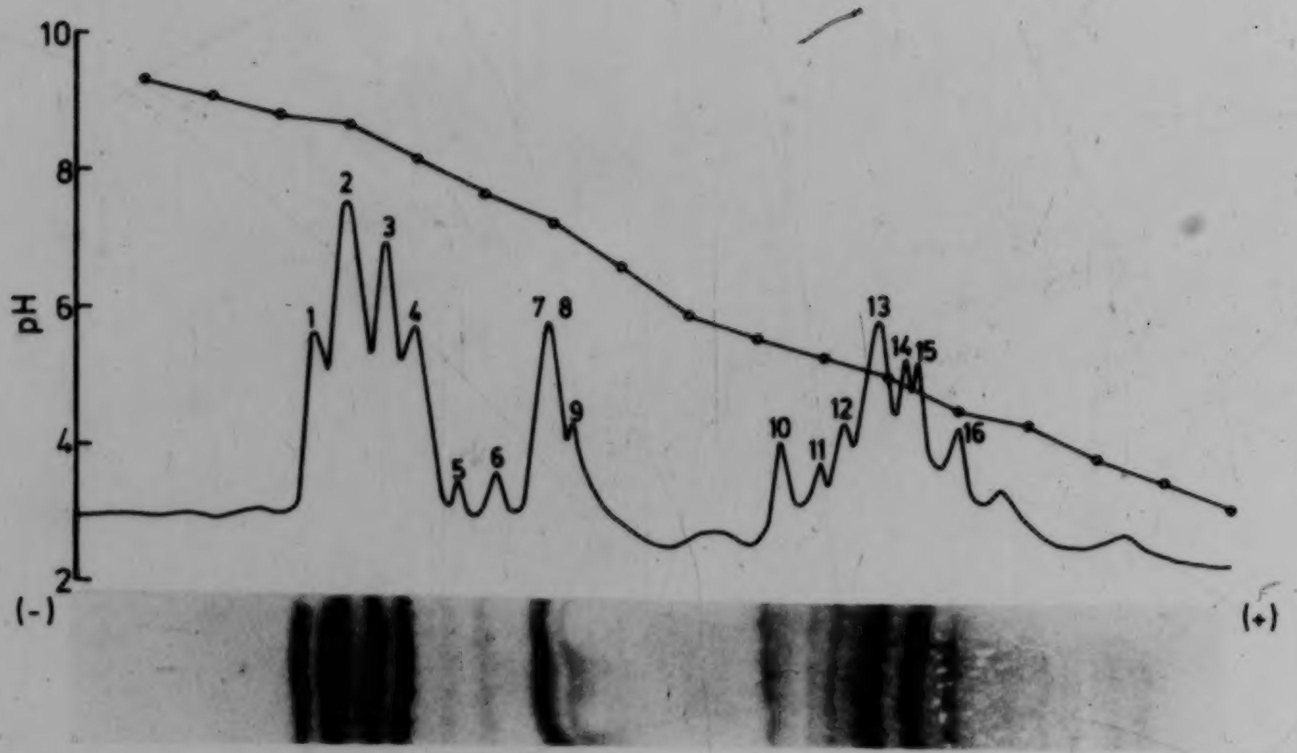


Figure 17. Scan of negative film picture of electrophoregram of potato sap proteins on TLIF, in pH 2-10 ampholytes. 565 µg protein was applied to the gel. pH gradient (○—○).

not complete. However, the water insoluble fraction was composed mainly of basic proteins, while the water soluble fraction covered a wider range of pHs, containing acidic and basic proteins.

In the sap proteins 16 distinctive bands were separated covering a similar pH range (4.5-8.6) as in the water soluble fraction. The major bands in the cathodic end were at pH's 8.6, 8.5, 8.2 and 8.0 (bands 1, 2, 3 and 4, Figure 17). Two major bands were at pH 7.0 region (bands 7 and 8, Figure 17). In the anodic end the most prominent protein bands were at pHs 4.9, 4.7 and 4.6 (bands 13, 14 and 15, Figure 17). TLIF of potato ('Netted Gem') sap proteins from different parts of the tuber OL, IL and whole tuber, a, c and d, respectively in Figure 18, showed no differences in pattern, but slight variations in band intensity. The water soluble and insoluble fractions (Figures 15 and 16) showed together a pattern similar to that obtained in the sap proteins. This indicates that qualitatively the sap proteins give a good representation of the major proteins of the potato. The smaller number of proteins recovered in the water soluble and insoluble fractions in comparison to the sap proteins may reflect changes in charge on the proteins caused in the extraction steps, mainly attributed to amides hydrolysis which is favored by high pH and phosphate ions (Stegemann *et al.*, 1973; Leach, 1953).

4.1.3.3. Amino Acid Analysis of Water Soluble and Insoluble Fractions

The common amino acids found in total amino acid analyses of potato powder were also detected in the extracted protein fractions; in addition cystine was detected in small quantity (Table 7). Due to the instability of cystine during acid hydrolysis no quantitative

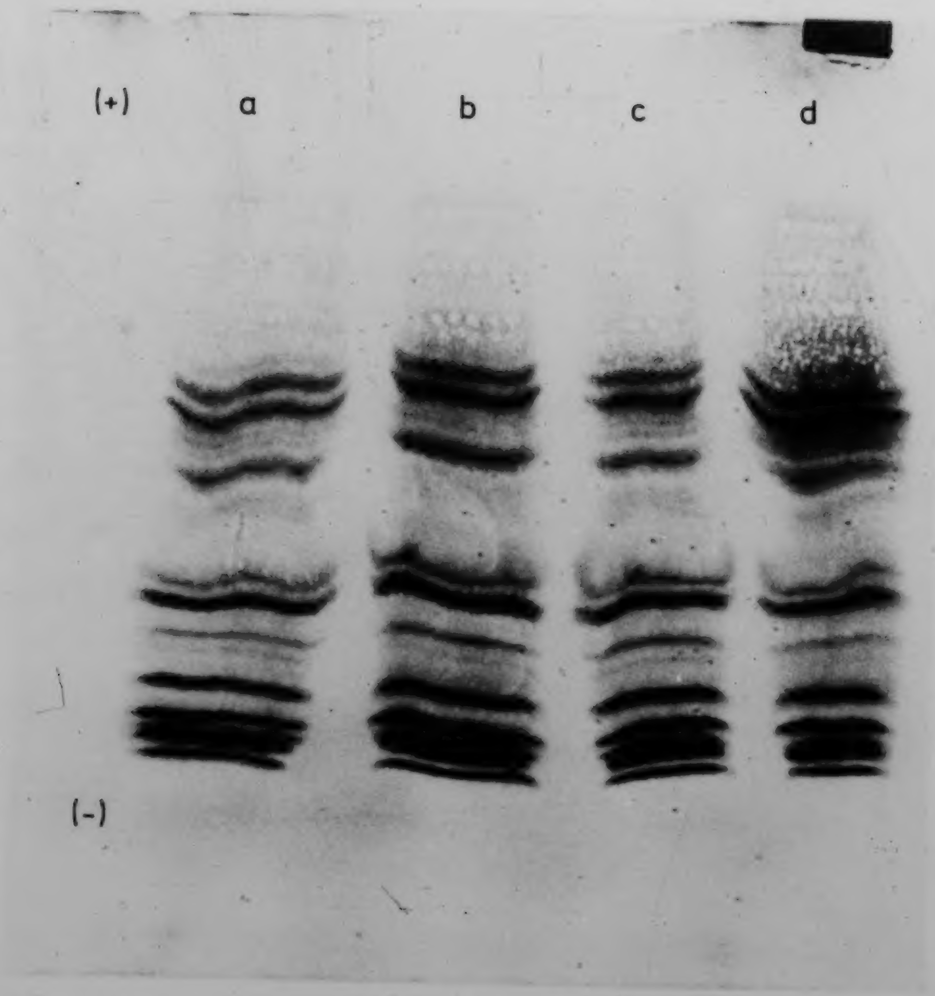


Figure 18. Electrophoregram of potato sap proteins on TLIF in pH 2-10 ampholytes. Inner layer (a), outer layer (b) and whole tuber (c,d). Approximately 1 mg protein per sample was applied to the gel.

Table 7. Amino acid composition of potato ('Netted Gem') water soluble and insoluble proteins.

Protein / Amino acid	Water soluble		Water insoluble	
	mg/g dry weight	wt/wt %*	mg/g dry weight	wt/wt %
Asp**	115.78	12.0	103.62	11.9
Thr	53.47	5.5	43.60	5.0
Ser	52.39	5.4	40.35	4.6
Glu**	109.21	11.3	91.03	10.4
Pro	51.51	5.3	45.74	5.2
Gly	48.88	5.1	42.13	4.8
Ala	47.26	4.9	37.84	4.3
Cys$_2$***	8.73	0.9	7.34	0.8
Val	61.17	6.3	60.59	6.9
Met	18.18	1.9	19.04	2.2
Ile	48.10	5.0	46.68	5.3
Leu	87.48	9.1	80.51	9.2
Tyr	56.02	5.8	46.59	5.3
Phe	61.93	6.4	54.67	6.3
His	21.94	2.3	22.66	2.6
Lys	78.93	8.2	79.92	9.2
Arg	44.85	4.6	51.14	5.9
Total	965.83	100.0	873.45	99.9

Results are the average of three determinations, average % CV = 6.

*Value represents the acid and the corresponding amide.

$$**\text{Wt/wt } \% = \frac{\text{mg amino acid} \times 100}{\text{total weight of amino acids}}$$

***Expressed as half cystine.

interpretation can be attempted, although the fact that cystine was detected in the extracted proteins and not in the whole potato powder implies that either the cystine was concentrated more in these fractions or that the purification step had removed a factor which contributed to the decrease of this amino acid. The high recovery of both water soluble and insoluble fractions, 96.6% and 87.3%, respectively, indicated that they were well purified. The larger proportion of basic amino acids (histidine, lysine and arginine) and lower proportion of acidic amino acids (aspartic and glutamic in the water insoluble fraction) (Table 7) may partially explain the high isoelectric points obtained for the major proteins in this fraction.

4.1.4. Column Chromatography of Water Soluble Proteins

The water soluble fraction of the potato ('Netted Gem') extracted as for the electrophoresis was further analyzed by column chromatography (gel filtration). The average recovery from the column was 94.2%.

The pattern of separation of the water soluble fraction of the inner layer of the tuber at maturity (Oct 28, harvest) is shown in Figure 19. A similar pattern of 4 peaks was obtained throughout the growing season as well as in proteins extracted from the outer layer of the tuber (Figure 20). The change in the relative amount of the different fractions during the season is shown in Table 8. There was no obvious trend of change during growth. Nevertheless, it was noted that the difference between the higher molecular weight fraction (A) >160,000, and the low molecular weight fraction (D) <13,000 has narrowed from the first to the last harvest (Table 8). This may indicate a trend of increase in larger molecular weight proteins as

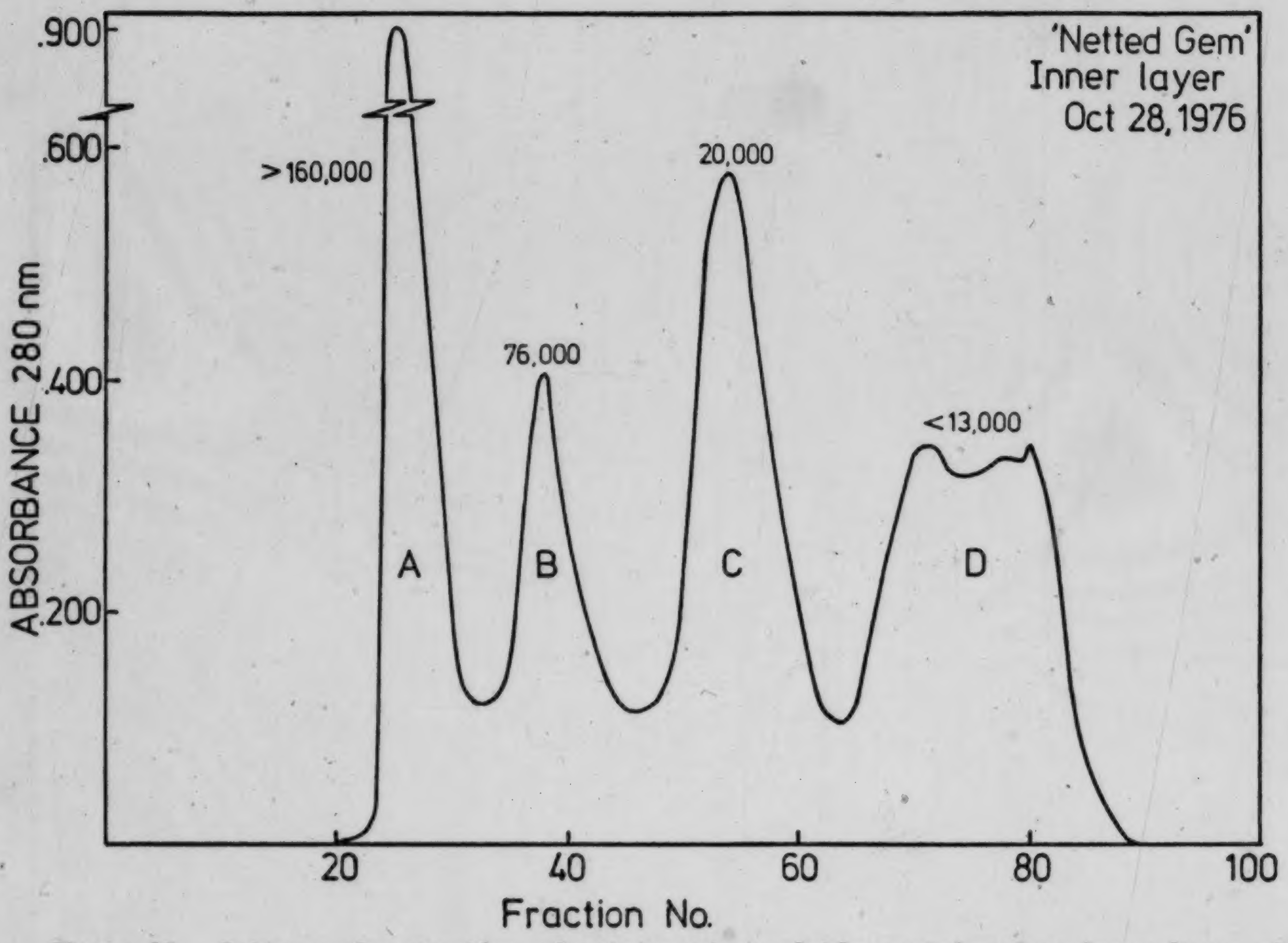


Figure 19. Pattern of separation of potato water soluble proteins from inner layer of mature tuber, obtained by column chromatography on Sephadex G-150. Molecular weight estimates are given beside the peaks. 88.5 mg protein was applied to the column.

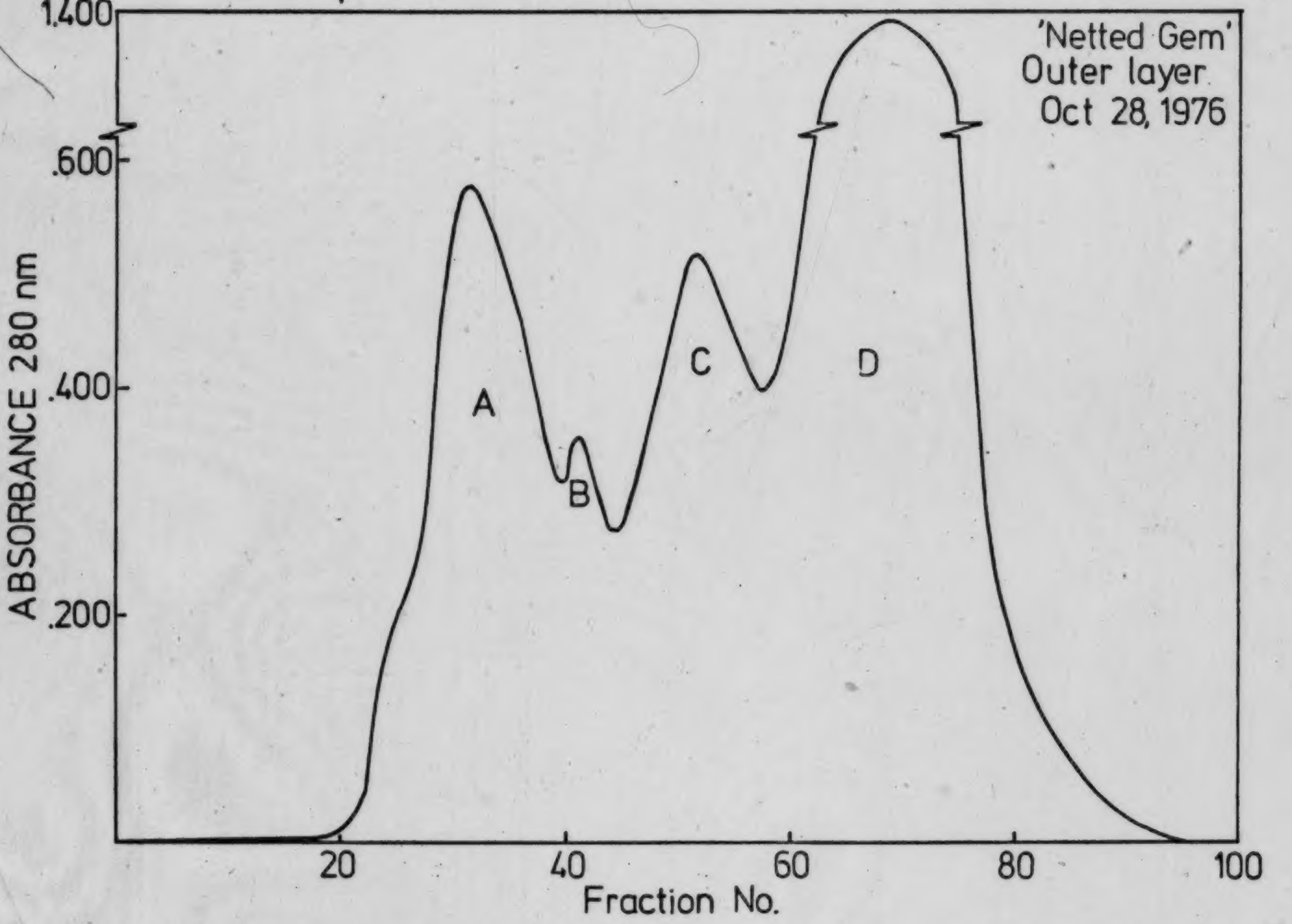


Figure 20. Pattern of separation of potato water soluble proteins from the outer layer of mature tuber obtained by column chromatography on Sephadex G-150. 85 mg protein was applied to the column.

Table 8. Potato ('Netted Gem') water soluble protein fractions during tuber development, analyzed by column chromatography on Sephadex G-150, and measured by absorption at 280 nm.

Fraction Harvest date	A	B	C	D
	Percent of total recovery*			
July 27	9.4	19.9	22.7	48.1
August 16	7.3	15.5	26.9	50.0
August 31	13.3	16.3	22.8	47.6
September 9	12.1	16.8	31.3	39.8
September 24	8.9	14.4	24.2	52.5
October 28**	21.9	17.0	28.7	32.3
October 28***	22.8	4.0	18.5	54.7

*Each protein fraction (A, B, C and D) at every harvest date is expressed as a percent of total protein recovered.

**Inner layer at maturity.

***Outer layer at maturity.

storage material. Fraction B in the OL of the tuber was significantly smaller compared to the other fractions, while fraction D appeared very large (Figure 20), when measured at 280 nm, but using the more specific protein determination by Lowry's method (Appendix 2) smaller amount of protein was determined. It indicates that this fraction may contain compounds other than proteins, or small peptides which absorb at 280 nm.

4.2. *Amino Acid Analysis*

4.2.1. Amides Determination by Ion-Exchange Chromatography

The method developed for amides determination is based on the hydrolytic conversion of asparagine and glutamine to the corresponding aspartic and glutamic acids. The Beckman 120B amino acid analyzer was used for analysis and only the long column was operated. The position on the chromatogram of the unresolved peak of standard amides (asparagine and glutamine) mixture is shown in Figure 21a. Chromatogram of an aliquot of the same standard mixture after elution from the column and hydrolysis (Figure 21b), shows the positions of the corresponding aspartic and glutamic acids. The unresolved amides peak in potato FAA extract is shown in Figure 22. The large amides peak overlaps completely serine and partially threonine peaks. The fraction corresponding to the unresolved peak was collected and hydrolysed as described in Section 3.5.2.2. An aliquot of the hydrolysed sample was rechromatographed (Figure 23). Aspartic and glutamic acid of the second run were used to determine the amides content in the original sample. The small peaks of threonine and serine can also be seen (Figure 23) between aspartic and glutamic acid peaks.

In preliminary experiments conditions for fast amides hydrolysis

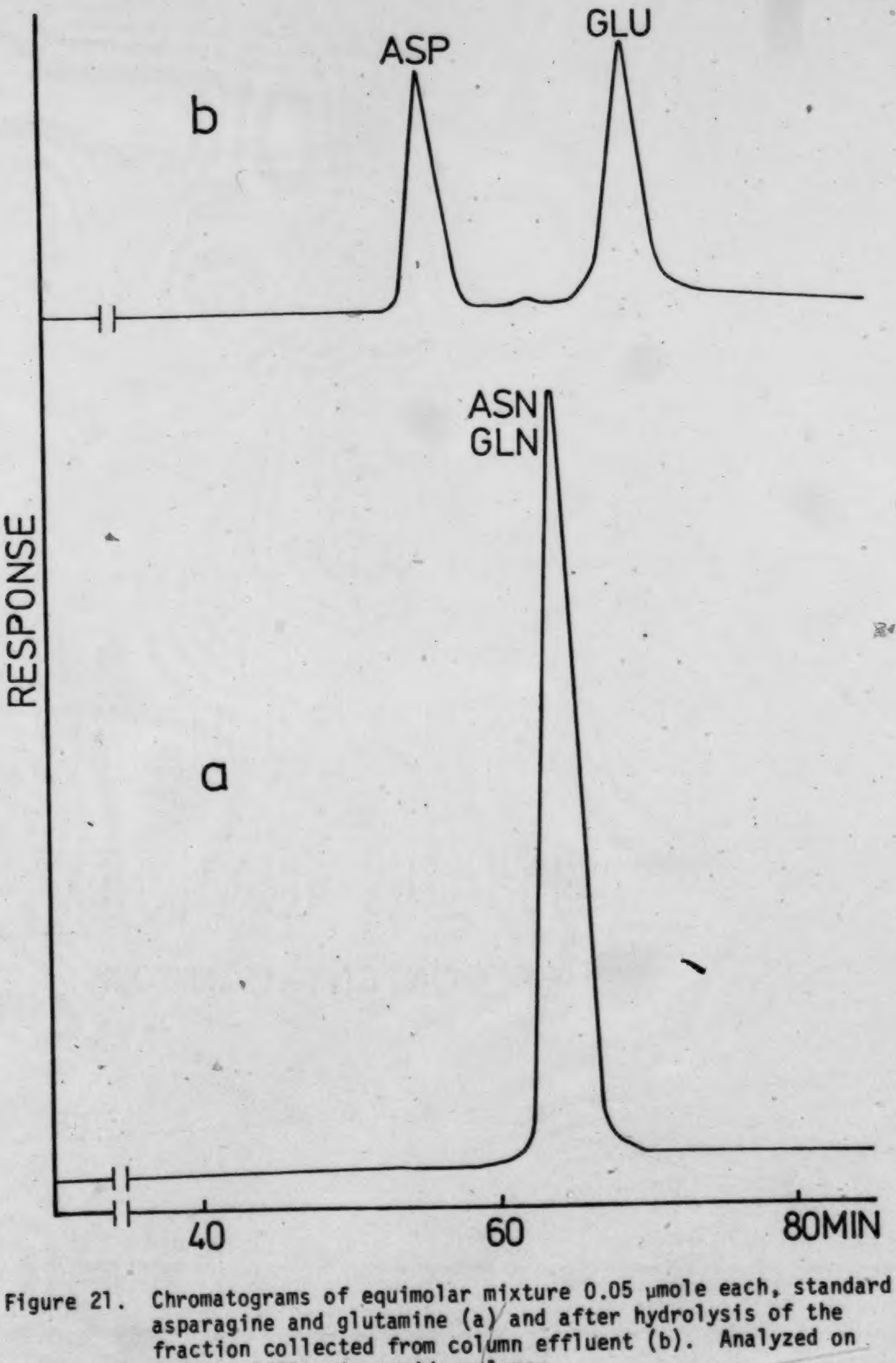


Figure 21. Chromatograms of equimolar mixture 0.05 µmole each, standard asparagine and glutamine (a) and after hydrolysis of the fraction collected from column effluent (b). Analyzed on Beckman 120B amino acid analyzer.

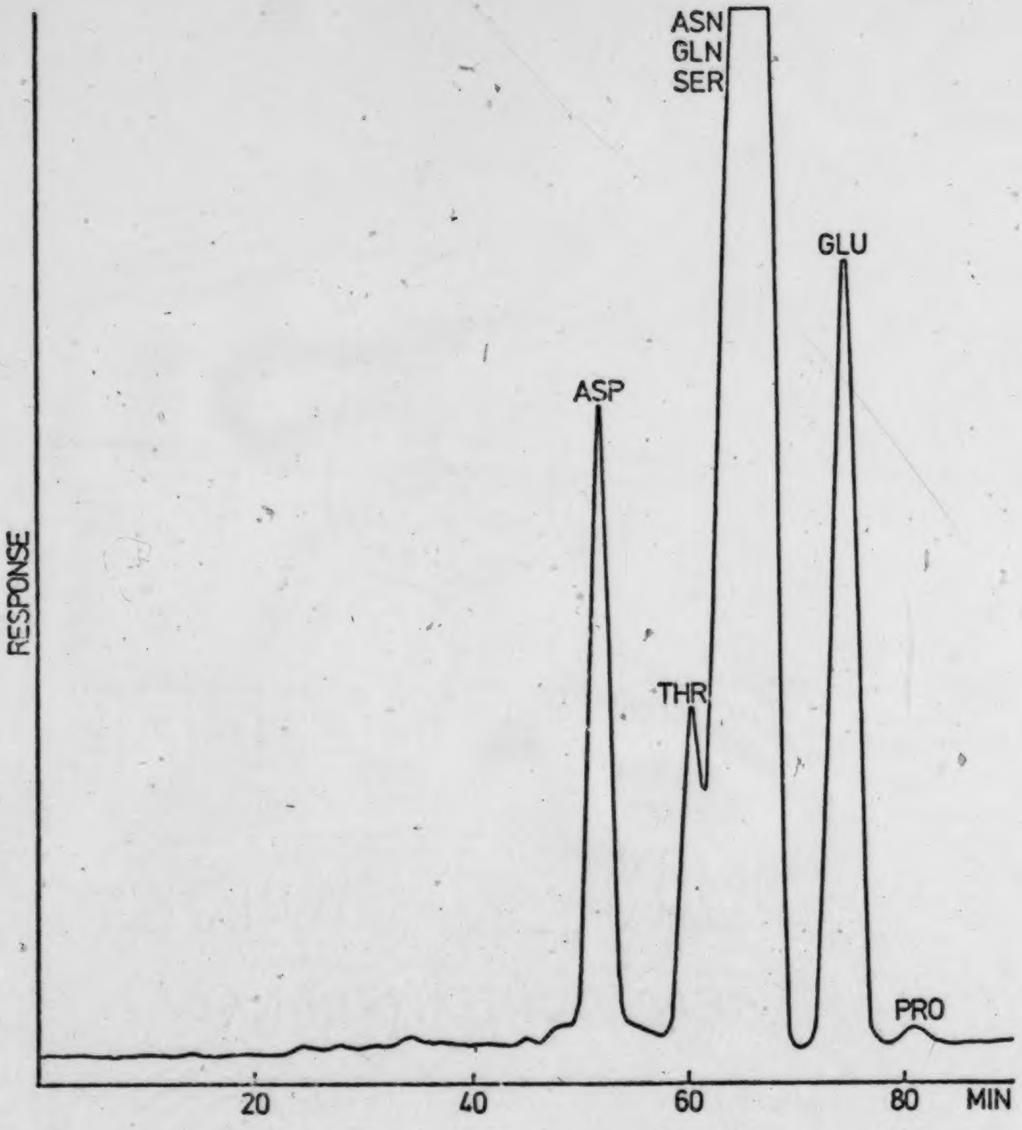


Figure 22. Chromatogram of potato free amino acid extract, analyzed on Beckman 120B amino acid analyzer. Representing 125 mg potato (fresh weight).

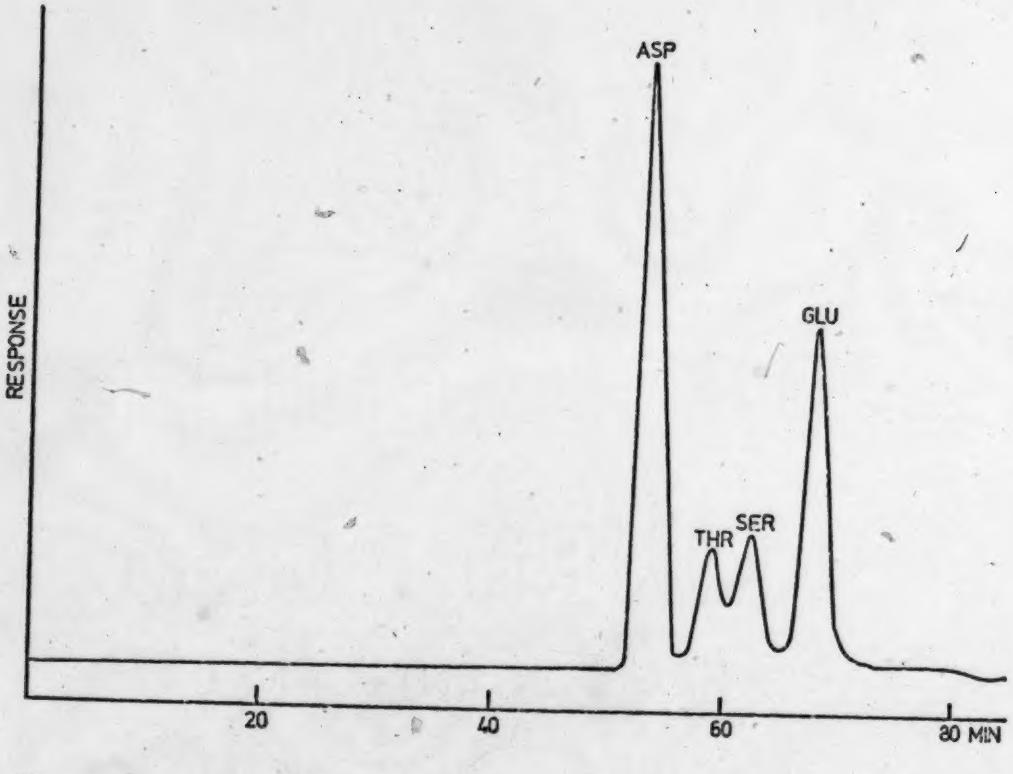


Figure 23. Chromatogram of hydrolysed potato FAA amides fraction, collected from column effluent. Analyzed on Beckman 120B amino acid analyzer. Representing 25 mg potato (fresh weight).

were evaluated. Mixtures of standard asparagine and glutamine were subjected to hydrolysis in concentrated hydrochloric acid (~12N) at room temperature. The hydrolysis was not complete even after 40 hrs. In Table 9 it is shown that upon hydrolysis at 130 ± 5°C in concentrated HCl maximum conversion of the amides to the corresponding amino acids was within 15 min. Duncan's multiple range test (Duncan's test) showed no significant differences (1% level) at longer hydrolysis time. Recoveries were high, 99.3% and 96.2% for asparagine and glutamine, respectively. Standard aspartic and glutamic acids subjected to the same treatment gave similarly complete recovery. The use of this procedure for amides determination in FAA extract of potato is presented later (Section 4.3.1.2.).

The effect of storage temperature (room temperature, ~23°C; refrigeration, ~1°C; freezer, ~-16°C), on amides stability in 0.1N HCl was evaluated. Figures 24, 25 and 26 show the effect of storage temperature on the stability of asparagine, glutamine and their mixture, respectively. The degradation of the amides is most significant at room temperature. The greatest degradation occurred within the first five days of storage. Glutamine showed a higher amount of degradation at all conditions. The degradation of the amides resulted in the release of ammonia and formation of the corresponding amino acids; on glutamine chromatograms the molar response of the glutamic acid formed was low. This may be due to cyclization of glutamic to pyrolidone-carboxylic acid. It has been shown to be a favorable reaction of glutamine under mild acidic conditions (Greenstein and Winitz, 1961).

Table 9. Percent recovery of asparagine and glutamine after hydrolysis in ~12N HCl at 130 ± 5°C.

Amide / Time (min)	Asparagine	Glutamine
	% Recovery*	
15	100.1^a	97.4^b
30	103.8^a	99.3^b
60	94.9^a	91.8^b

*Calculated from molar response of corresponding amino acids relative to the amount of amide subjected to hydrolysis.

(a,b)- A common letter on a sequence of means in a column indicates that there is no significant difference at 1% level (Duncan's test).

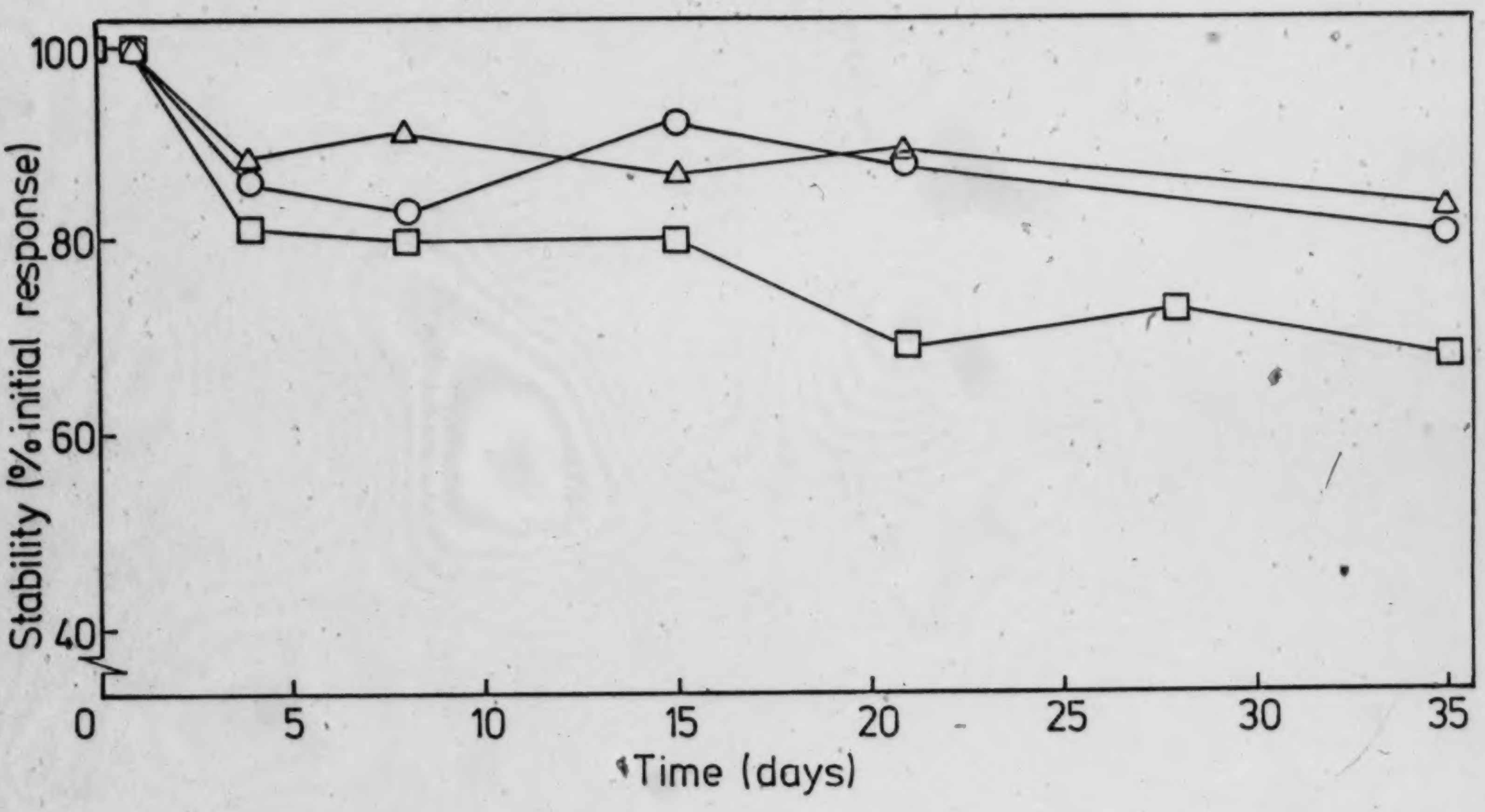


Figure 24. Effect of storage temperature on asparagine stability in 0.1N HCl solution. Room temperature, ~23°C (□); refrigeration temperature ~1°C (○); freezer temperature ~-16°C (△).

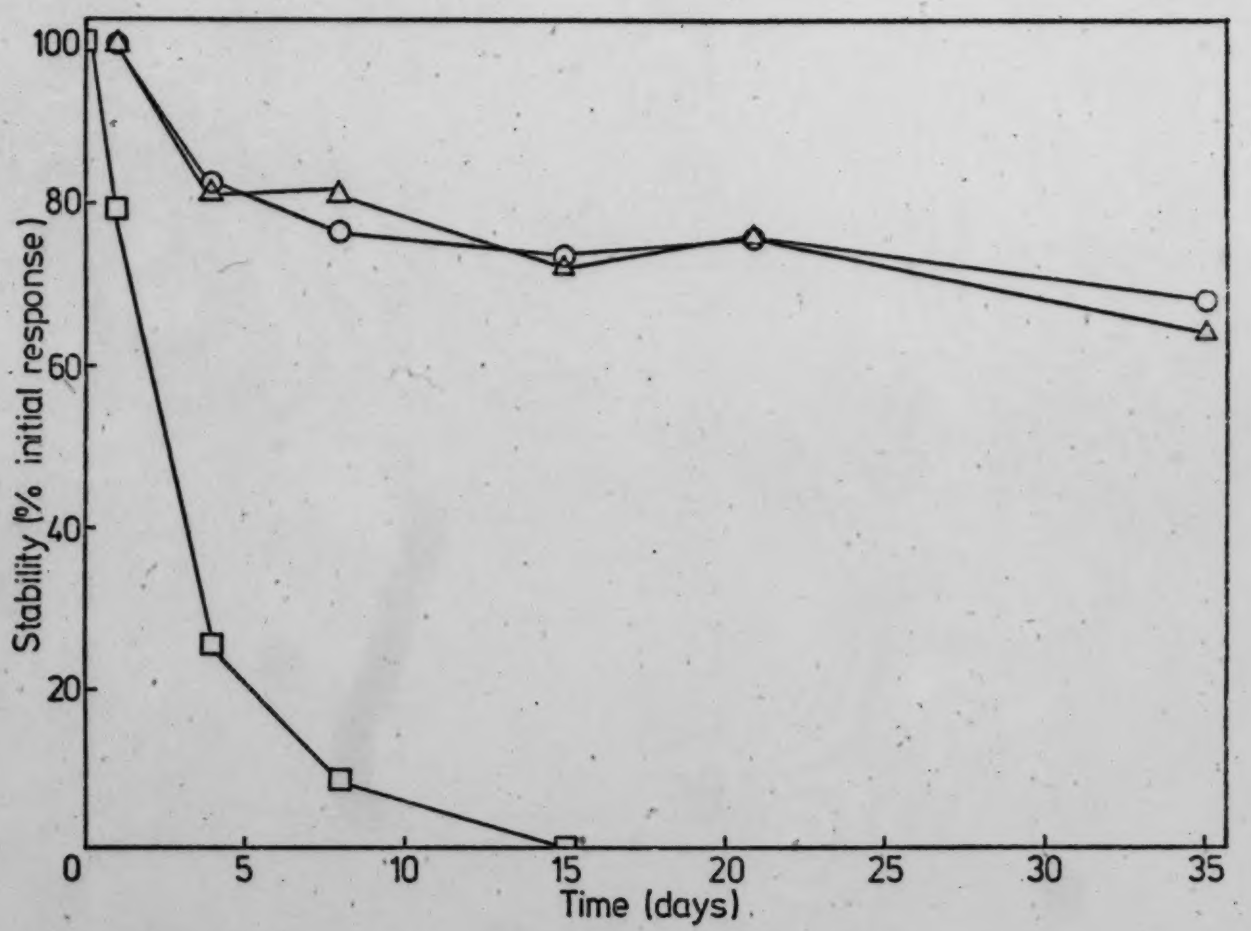


Figure 25. Effect of storage temperature on glutamine stability in 0.1N HCl solution. Room temperature, ~23°C (□); refrigeration temperature ~1°C (○); freezer temperature ~-16°C (△).

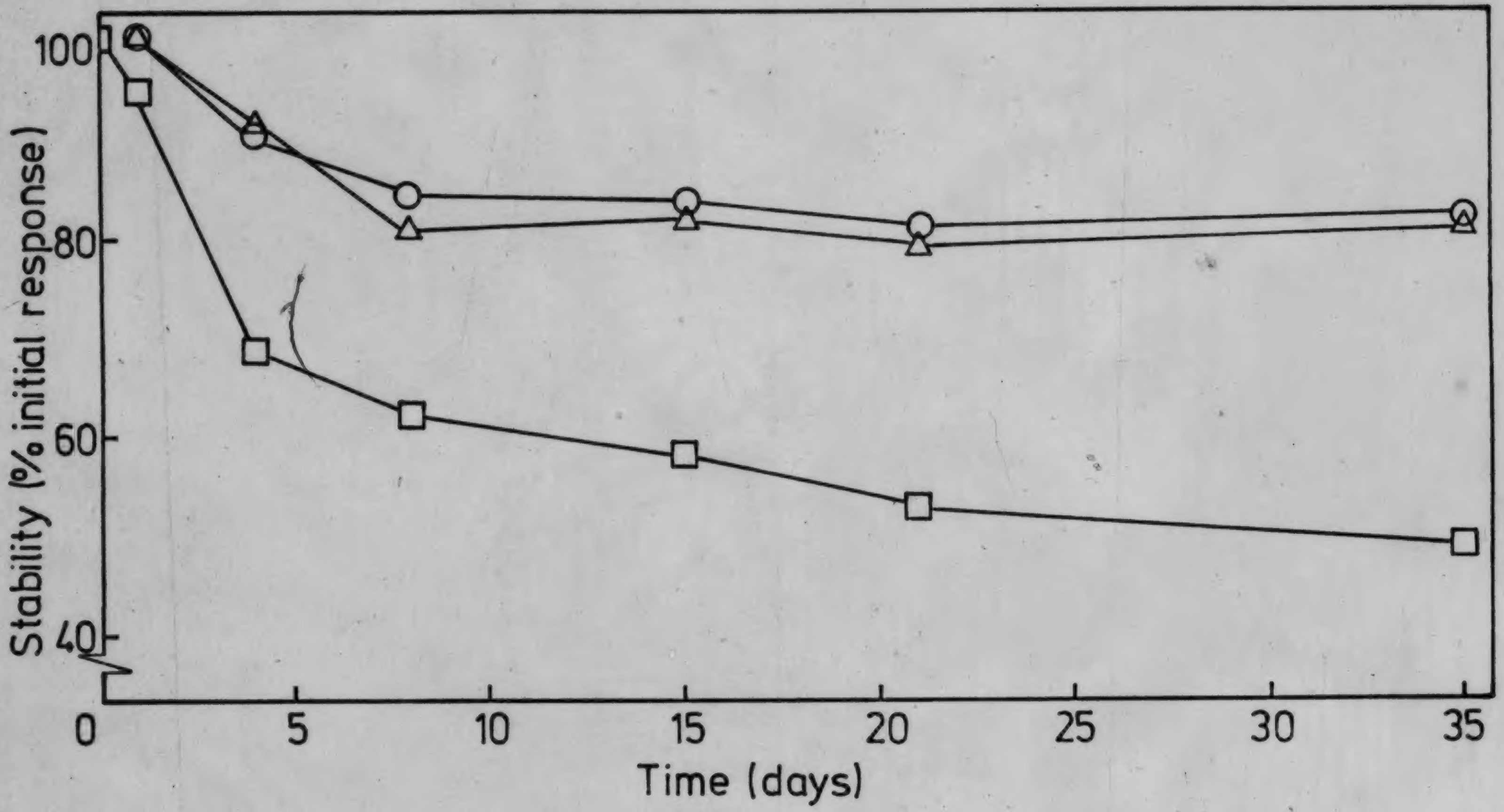


Figure 26. Effect of storage temperature on stability of asparagine and glutamine mixture in 0.1N HCl solution. Room temperature ~23°C (□); refrigeration temperature ~1°C (○); freezer temperature ~-16°C (△).

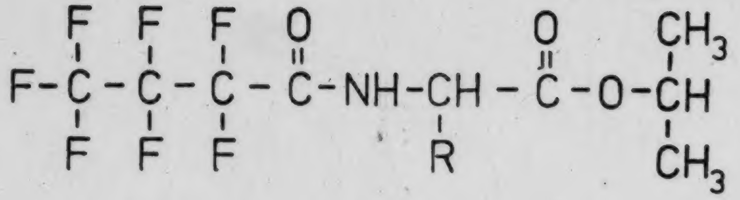
4.2.2. Gas Chromatographic Analysis of Amino Acids

4.2.2.1. Pattern of Separation

The pattern of separation of N-HFB-isopropyl ester derivatives (Figure 27) of standard amino acid equimolar mixtures is shown in Figure 28a,b. The apparent resolution was not improved using the slower temperature programming rate, 8°C/min (Figure 28b). Hence, the faster temperature programming rate, 20°C/min (Figure 28a) was used for routine amino acid analysis, as it provided a significant reduction in analysis time. γ-aminobutyric acid (GABA) was added to the standard amino acid mixture. Figure 29 shows the profile of separation after derivatization, and reveals the position of GABA immediately after isoleucine. The order of elution of the N-HFB-isopropyl ester derivatives was similar to that of the analoguous derivatives esterified with *n*-propanol (Moss *et al.*, 1971) and isobutanol (Mackenzie and Tenaschuk, 1974). The only differences were in methionine eluting well resolved after aspartic acid and glutamic acid eluting before phenylalanine. The resolution between lysine and tyrosine became poorer after over hundred analyses on the same column.

4.2.2.2. Derivatization

In preliminary study it was found that higher relative molar response (RMR) of most of the derivatives was obtained when esterification was done at 80°C for 120 min and acylation at 110°C for 10 min (Appendix 3). These conditions were used for routine amino acid analysis. The long esterification time was not a limiting factor because, usually, samples were processed in batches. Ornithine was selected as an internal standard, as it is non-protein amino acid, well separated from other



$$CF_3-CF_2-CF_2-C(=O)-NH-CH(R)-C(=O)-O-CH(CH_3)_2$$

Figure 27. Formula of N-heptafluorobutyryl-isopropyl ester derivative of amino acid. Functional groups (NH_2, OH and SH) on side chain (R) are acylated. Carboxyl groups are esterified.

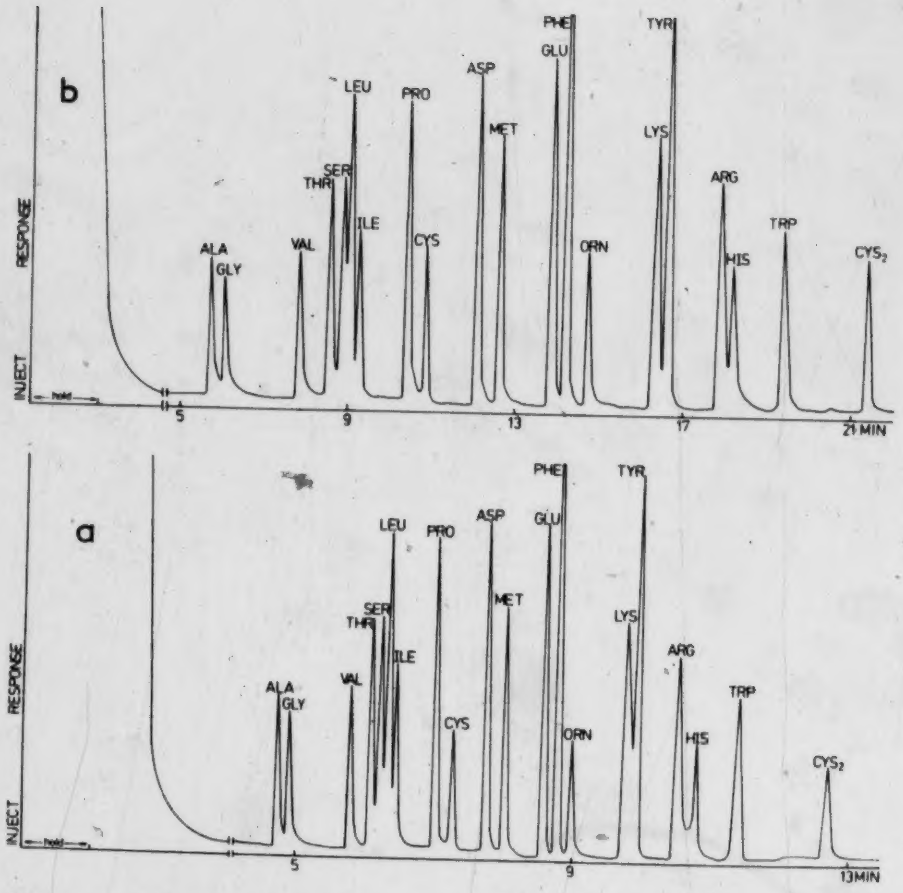


Figure 28. Gas-chromatogram of N-HFB-isopropyl ester derivative of standard amino acid mixture temperature program: 70°C 1 min, then temperature programming at 20°C/min (a), 8°C/min (b) to 225°C and hold at 225°C. Chart speeds, 100 inch/hr (a) and 60 inch/hr (b). Sample size approximately 6.5 nmoles. Cys, Cys_2 and ornithine 3.25 nmoles each. Internal standard ornithine.

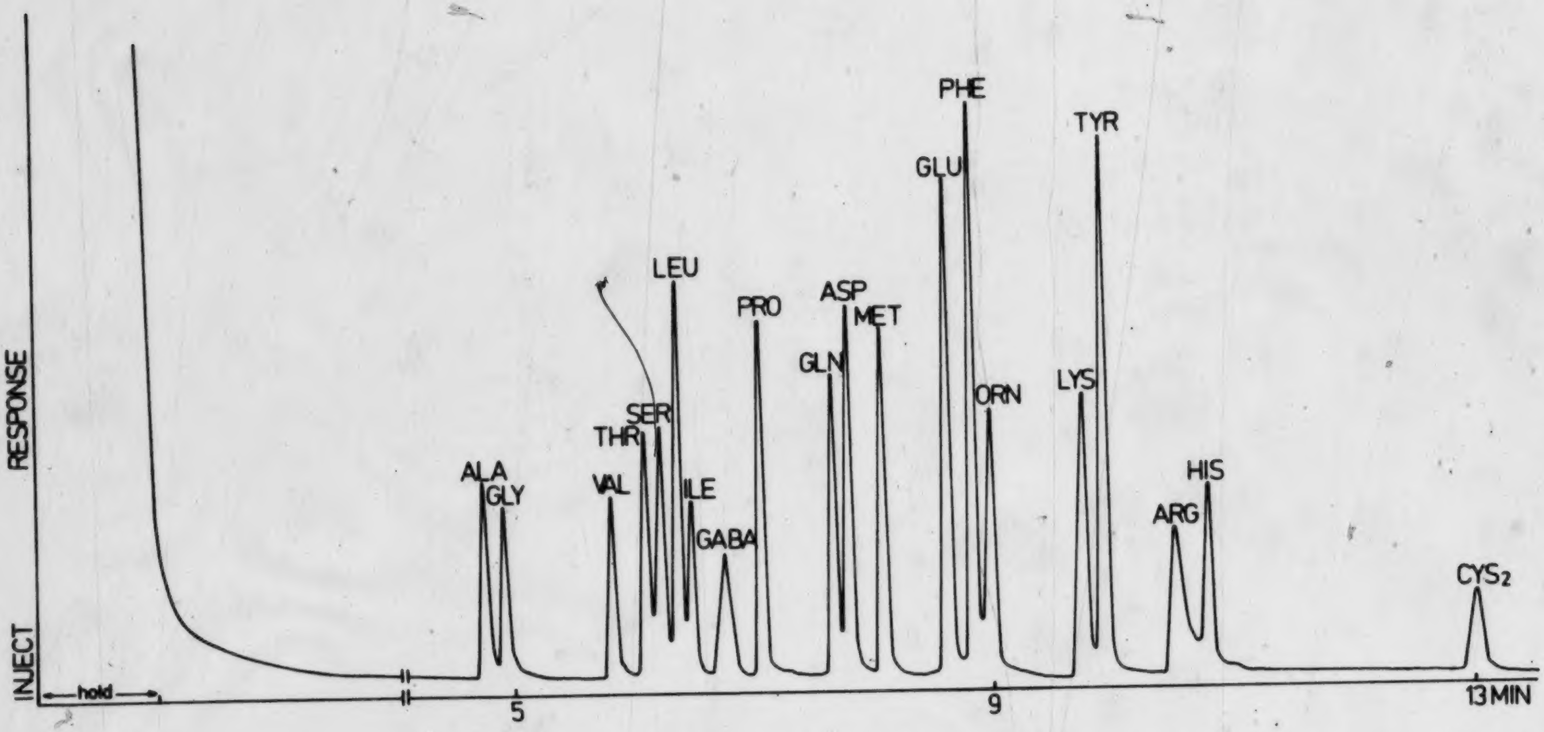


Figure 29. Gas-chromatogram of N-HFB-isopropyl ester derivative of standard amino acid mixture containing γ-aminobutyric acid (GABA). Temperature program: 70° 1 min, then temperature programming at 20°C/min to 225°C and hold at 225°C. Sample size approximately 5 nmoles. Cys$_2$ and ornithine 2.5 nmoles. Internal standard ornithine.

protein amino acids. Characteristic relative molar response (RMR) values based on 24 chromatograms and at least 8 replicates for each amino acid are given in Table 10. The average deviation of RMR values was approximately 9.5%. This deviation was mainly attributed to the low precision of peak area measurement by the manual integration method used (Gill, 1973). Peaks with a tailing end (glycine, arginine and histidine) were most difficult to measure accurately. Histidine, in addition, was less stable in storage at -16°C compared to other amino acid derivatives. Lower RMR values for alanine, glycine and valine were also obtained by other workers for similar acylalkyl derivatives (Roach and Gehrke, 1969; Kirkman, 1974; Pearce, 1977). This may be due to losses at the evaporation steps, especially with the more volatile N-HFB-isopropyl ester derivatives. In spite of the long esterification time the RMR of isoleucine was low (0.461). It is known that the esterification rate of all secondary alcohols is slower (Brown, 1975). Roach and Gehrke (1969) have shown that by increasing the esterification time higher RMR of N-trifluoroacetyl-*n*-butyl isoleucine was obtained, but degradation of some other derivatives occurred. As well, the long side chain of isoleucine could probably be involved in steric hindrance in the esterification reaction.

The alternative esterification reagent (see Section 3.6.1.) was mostly used as it offered a real advantage in time saving for reagent preparation. RMR values obtained by this method (Table 10) were closely comparable to those obtained by the regular esterification procedure (Appendix 3). The differences could be attributed to: 1. the different reagents, and 2. the different integration methods used

Table 10. Characteristic relative molar response (RMR) of N-HFB-isopropyl ester derivatives for quantitative determination of amino acids.

Amino acid	RMR*
Ala	0.459 ± 0.026
Gly	0.430 ± 0.031
Val	0.473 ± 0.059
Thr	0.738 ± 0.078
Ser	0.809 ± 0.092
Leu	1.043 ± 0.120
Ile	0.461 ± 0.059
GABA	0.543 ± 0.065
Pro	0.916 ± 0.090
Asp	0.988 ± 0.074
Met	0.725 ± 0.051
Glu	1.101 ± 0.073
Phe	1.429 ± 0.160
Lys	1.079 ± 0.051
Tyr	1.313 ± 0.121
Arg	0.821 ± 0.129
His	0.639 ± 0.063
Trp	0.730 ± 0.064
Cys_2	0.875 ± 0.065

*Internal standard ornithine.

in evaluating the peak areas. The quantitative determination by the GLC method was based on the reproducibility of the RMR values, therefore standard amino acid mixture was always analyzed with any set of unknown samples under identical conditions.

4.2.2.3. Retention Time Reproducibility

Complete elution of the N-HFB-isopropyl ester derivative of amino acids was achieved within 11-14 min. Relative retention time (RRT) is shown to be a preferable measure for evaluation of the pattern of separation than retention time (RT) (Table 11). This was expressed by the lower % CV values for RRT compared with RT. Comparison of RRT of protein hydrolysates and standard amino acid derivatives on another column (Table 12) showed high reproducibility of the pattern of separation.

4.2.2.4. Applicability of the Method

Evaluation of the N-HFB-isopropyl ester derivative of amino acids was accomplished by comparison of the GLC procedure with an established method, IEC. An hydrolysate of BSA was subjected to analysis by both methods. The amino acid derivatives pattern of separation by GLC for BSA is shown in Figure 30.

The applicability of the GLC method to protein hydrolysate is obvious from the similarity of the relative mole % values obtained by both methods, considering the fact that proline was determined only by the GLC method (Table 13). The large % CV values calculated for the GLC compared to the IEC data may reflect the lower precision of manual compared to electronic integration, respectively. % CV estimates based on manual methods of peak area measurement are two to five times larger

Table 11. Retention time (RT) and relative retention time (RRT*) of N-HFB-isopropyl ester derivative of amino acids.

	RT (sec)			RRT		
AA	Mean	± SD	% CV	Mean	± SD	% CV
Ala	240	11.1	4.6	0.477	0.020	4.2
Gly	252	10.7	4.2	0.501	0.019	3.8
Val	307	7.4	2.4	0.610	0.012	2.0
Thr	327	6.3	1.9	0.650	0.009	1.4
Ser	335	6.1	1.8	0.666	0.009	1.3
Leu	341	5.9	1.7	0.678	0.008	1.2
Ile	349	7.2	2.1	0.694	0.011	1.6
Pro	383	4.9	1.3	0.761	0.006	0.8
Cys	399	4.1	1.0	0.793	0.004	0.5
Asp	429	3.8	0.9	0.853	0.004	0.5
Met	444	3.1	0.7	0.883	0.004	0.4
Glu	478	3.8	0.8	0.950	0.003	0.3
Phe	488	3.8	0.8	0.970	0.004	0.4
Orn	503	3.7	0.7	1.000	0	0
Lys	546	4.2	0.8	1.085	0.004	0.4
Tyr	553	3.7	0.7	1.100	0.003	0.3
Arg	587	3.8	0.6	1.167	0.003	0.3
His	600	9.0	1.5	1.193	0.016	1.3
Trp	639	3.4	0.5	1.270	0.004	0.3
Cys_2	705	4.8	0.7	1.402	0.006	0.4

*Relative to ornithine's retention time.

Table 12. Relative retention time (RRT)* of protein hydrolysates and Standard Amino Acids.

protein AA	Standard		Bovine serum albumin		Casein	
	mean	% CV	mean	% CV	mean	% CV
Ala	0.540	5.3	0.556	1.4	0.543	1.6
Gly	0.560	4.8	0.575	1.4	0.564	1.7
Val	0.652	3.4	0.664	1.0	0.635	6.8
Thr	0.684	2.7	0.690	0.7	0.678	0.6
Ser	0.702	2.3	0.704	0.5	0.694	0.4
Leu	0.714	2.3	0.719	0.6	0.707	0.5
Ile	0.725	2.3	0.730	0.7	0.720	0.4
Pro	0.789	1.7	0.792	0.5	0.785	0.3
Asp	0.864	1.0	0.868	0.5	0.858	0.3
Met	0.893	0.9	0.898	1.0	0.888	0.4
Glu	0.955	0.4	0.955	0.4	0.949	0.3
Phe	0.976	0.5	0.976	0.5	1.967	0.3
Lys	1.080	0.7	1.005	0.1	1.070	0.2
Tyr	1.090	0.8	1.015	0.1	1.080	0.4
Arg	1.159	0.9	1.150	0.2	1.139	0.4
His	1.191	1.0	1.200	0.1	1.185	1.1
Cys_2	1.435	1.7	1.430	0.5	1.324	4.9

*Relative to ornithine's retention time.

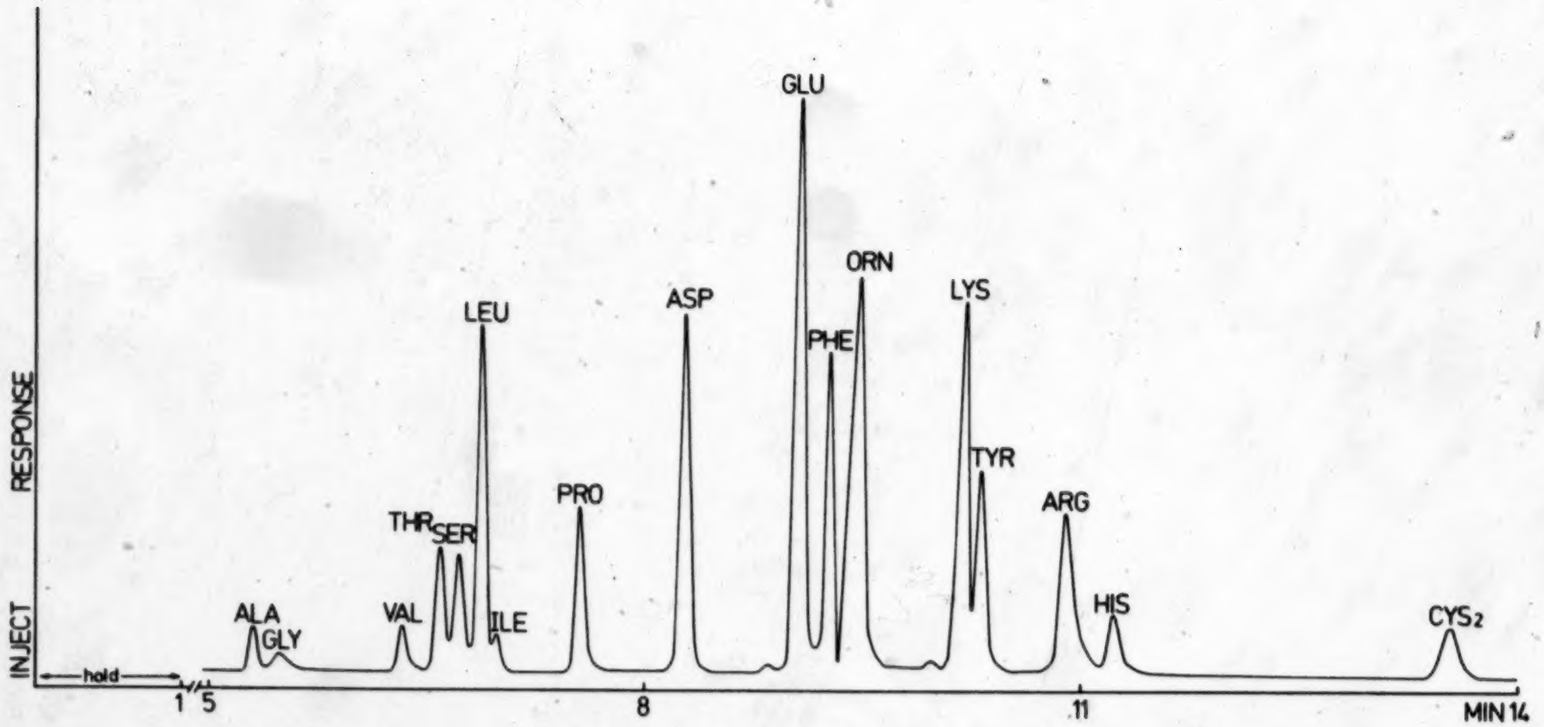


Figure 30. Gas-chromatogram of N-HFB-isopropyl amino acid derivatives of hydrolyzed bovine serum albumin. Representing approximately 18 μg BSA. Internal standard ornithine. Temperature program: 70°C 1 min, then temperature programming at 20°C/min to 225°C and hold at 225°C.

Table 13. Comparison of amino acid analysis of bovine serum albumin (BSA) by gas liquid chromatography (GLC) and ion-exchange chromatography (IEC).

Method / Amino acid	IEC		GLC	
	mean	% CV	mean	% CV
	mole %			
Ala	8.3	0.9	7.1	12.9
Gly	2.9	2.0	2.9	35.5
Val	6.5	2.5	4.7	15.1
Thr	6.0	2.4	5.9	3.5
Ser	4.6	2.2	5.0	2.7
Leu	11.2	1.9	10.6	14.7
Ile	2.4	1.8	2.2	13.5
Pro*	-	-	6.1	2.9
Asp	10.0	2.1	9.8	22.6
Met	0.7	1.6	0.5	30.0
Glu	14.8	4.3	14.5	13.7
Phe	4.9	2.2	4.7	9.9
Lys	10.9	4.5	10.0	9.3
Tyr	3.6	3.2	3.4	27.6
Arg	4.2	1.5	4.0	11.1
His	3.1	0.6	3.3	13.3
Cys_2**	5.5	4.4	5.0	12.5

*Proline chanel on IEC system not operated.

**Expressed as half cystine.

than those derived from electronic integration (Gill, 1973). The large deviation of methionine value in BSA by the GLC method (% CV = 30) may be attributed to the lower precision of peak area measurement for very small peaks (0.5 mole %).

In addition, an attempt was made to compare IEC to GLC, using a crude preparation of mixed caseins. In this case the clean-up step (Gehrke *et al.*, 1968; Adams *et al.*, 1977) was omitted. While the pattern of separation (Figure 31) was similar to BSA, there were significant differences in mole % as compared to IEC (Table 14). These differences may be attributed to impurities in the casein preparation. Hence, the clean-up step was included in the analysis of the potato free amino acid pools by GLC method.

4.2.3. Gas Chromatographic Analysis of Asparagine and Glutamine

Asparagine and glutamine (amides) analysis by GLC poses a special difficulty because they are partially hydrolysed under the acidic condition used for derivatization. When the pure amides were derivatized separately two peaks were obtained for each one of them (Figure 32, a and b). In each case, the first peak most probably corresponds to the non-hydrolysed amide and the second appears at the position of the corresponding amino acid. From the peak sizes it may be inferred that a substantial portion of each amide was hydrolysed to the acid. Analysis of the amides in a mixture of amino acids (Figure 33) showed that glutamine was well separated, while asparagine was poorly resolved from isoleucine, an improved separation of asparagine was obtained by conducting the analysis at reduced temperature programming rate (8°C/min). When a large amount of asparagine was

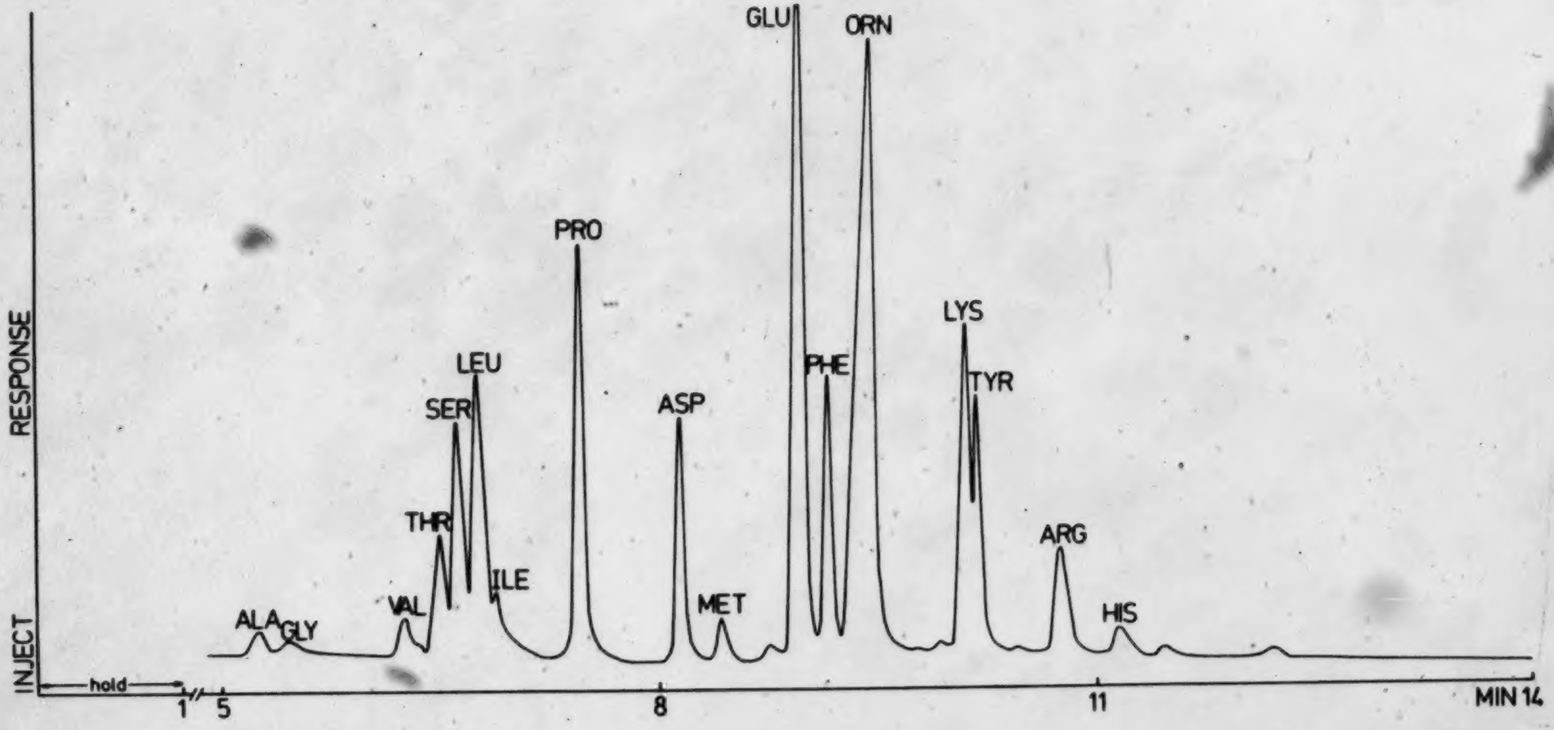


Figure 31. Gas-chromatogram of N-HFB-isopropyl amino acid derivatives of hydrolyzed casein. Representing approximately 25 μg casein. Internal standard ornithine. Temperature program: 70°C 1 min, then temperature programming at 20°C/min to 225°C and hold at 225°C.

Table 14. Comparison of amino acid analysis of casein by gas liquid chromatography (GLC) and ion-exchange chromatography (IEC).

Method Amino acid	IEC		GLC	
	mean	% CV	mean	% CV
	mole %			
Ala	4.7	4.3	2.6	16.6
Gly	3.6	5.8	2.2	23.5
Val	8.1	2.8	4.2	3.9
Thr	4.9	4.4	5.0	16.4
Ser	7.6	3.7	7.8	5.3
Leu	8.8	7.4	7.1	5.3
Ile	5.9	4.9	5.1	18.0
Pro *	-	-	11.4	4.7
Asp	7.7	4.2	7.4	19.5
Met	2.9	2.0	2.7	13.4
Glu	22.4	5.8	19.2	4.2
Phe	4.4	2.9	4.5	9.4
Lys	8.0	3.3	9.4	5.5
Tyr	4.5	2.3	5.8	11.6
Arg	3.1	3.1	2.5	1.3
His	2.7	1.8	2.6	18.8

*Proline chanel on IEC system not operated.

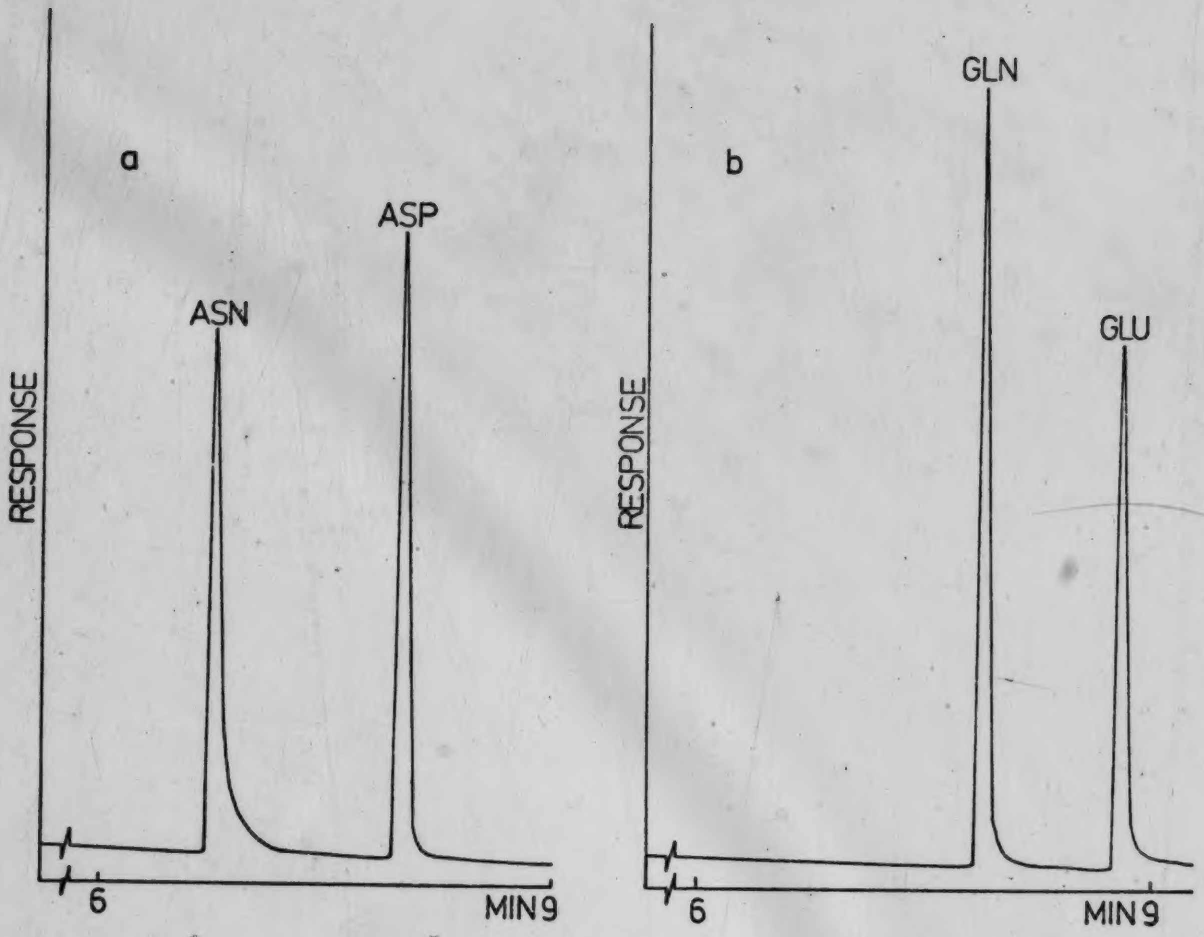


Figure 32. Gas-chromatogram of asparagine (a) and glutamine (b) derivatized separately. Temperature program: 70°C 1 min, then temperature programming at 20°C/min to 225°C and hold at 225°C.

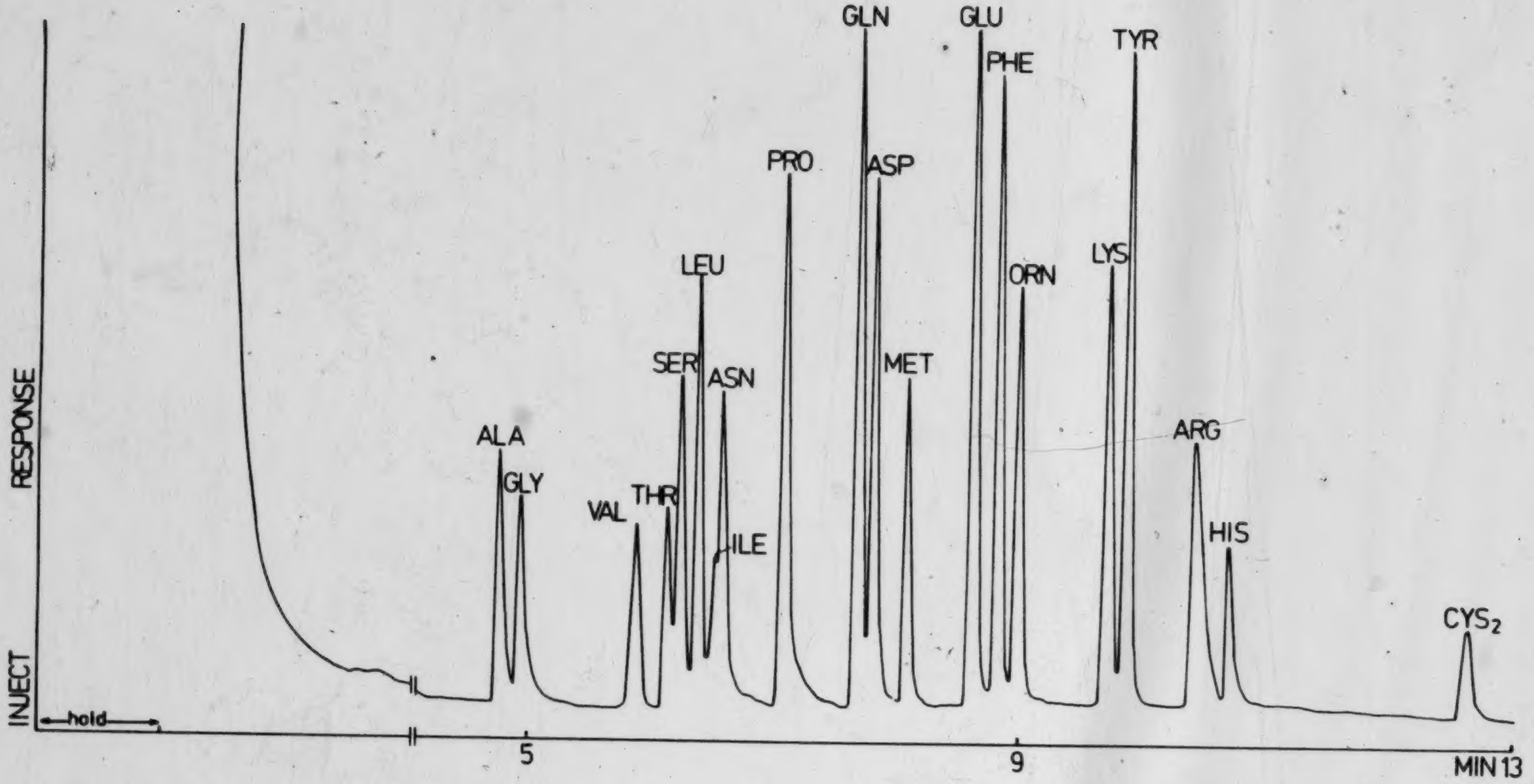


Figure 33 . Gas-chromatogram of N-HFB-isopropyl ester derivative of standard amino acid mixture containing asparagine and glutamine. Temperature program: 70°C 1 min, then temperature programming at 20°C/min to 225°C and hold at 225°C. Internal standard ornithine.

present in a sample, even at reduced temperature programming rate, the resolution of asparagine was poor. Derivatization of the amides in a mixture of amino acids resulted in an apparent difference in the amount of conversion of the amide to the acid, as compared to the situation when pure standard amides were derivatized. In addition, the glutamine derivative was not stable, its response decreased to half within 5 days. Asparagine showed higher stability.

Due to these difficulties quantitative determination of the amides and their corresponding amino acids in the FAA pool of the potato had to be done using the IEC method (see Section 3.5.2.).

4.3. *Changes in Total Proteins and Free Amino Acids During the Add-Back Process*

4.3.1. General Composition

Samples collected from the processing line in Vauxhall Foods Ltd. (Vauxhall, Alberta, Canada) (see Figure 3) were used for free amino acids (FAA) extraction. Dry matter, total nitrogen and FAA content at different stages of the add-back (A-B) process are given in Table 15. The changes in dry matter were in general agreement with the process specifications. There were significant (1% level) changes in total nitrogen content at the pre-cooking step. The significant loss at this stage was probably due to leaching of nitrogen containing solutes into the cooking water. The decrease in nitrogen content at the mash-mixing step might reflect the basic characteristics of the A-B process, where the final granules with lower nitrogen content are added-back in a ratio of 2:1 to 2.5:1 to the freshly cooked potatoes. In addition, losses at the cooling and steam cooking step might have also occurred. The changes in the subsequent steps were gradual and not significant. Total nitrogen content in the 'scalp' did not differ significantly from that in the raw potato. However, it is of nutritional importance, because the 'scalp' is used for animal feed. There was a significant decrease in FAA content, as well as at the pre-cooking step (Table 15).

The slight increase in total nitrogen and FAA at the air-lift drying stage was not significantly different from the other steps and within the experimental error (Table 15). However, change in the physical properties of the granules at this stage could have affected the efficiency of the extraction with 70% ethanol. FAA content of the 'scalp' was not different from that of the final granules but both were significantly different from the amount found in the peeled raw potato tuber.

4.3.1.2. Amides Analysis

The substantial amounts of amides in the FAA pool of the potato and the difficulty encountered in their quantitative determination by the GLC method has prompted the development of a simple procedure for their direct determination based on ion-exchange chromatography.

Each potato sample was applied to the chromatographic column twice. In the first run the original aspartic acid and glutamic acid were determined and the amides collected from column effluent. The fraction collected was hydrolysed as described in Section 3.5.2.2. and rechromatographed after drying and hydrolysis for the determination of the resultant aspartic and glutamic acids. The changes in the amides and corresponding amino acids of the FAA pool during the add-back process are given in Table 16.

Table 15. Changes in potato ('Netted Gem') composition during the add-back process for potato granules production.

Processing step	Percent dry matter	% nitrogen of dry matter	Free amino acids µmole/g dry weight
Peeled raw potato	21.88 ± 2.36	1.8^{b}	249.53^{b}
After pre-cooking	21.29 ± 2.09	$1.5^{a.b}$	$206.00^{a,b}$
After mash-mixing	72.22 ± 0.82	1.3^{a}	145.60^{a}
After conditioning	75.48 ± 0.07	1.1^{a}	148.90^{a}
After air-lift drying	86.40 ± 0.45	1.2^{a}	$173.90^{a,b}$
Final product	94.65 ± 0.03	1.1^{a}	157.57^{a}
'Scalp'	87.00 ± 2.72	$1.5^{a,b}$	133.97^{a}
Average % CV	3.64	10.2	15.9

(a,b) A common letter in a column of means indicates that there is no significant difference at 1% level (Duncan's test).

Table 16. Changes in amides and corresponding amino acids in the free amino acid pool of the potato ('Netted Gem') during the add-back process.

Processing step / Amino acid or amide	Aspartic acid	Glutamic acid	Asparagine	Glutamine
	mg/g dry weight			
Peeled raw potato	1.02 ± 0.20	2.18 ± 0.04	8.83 ± 1.63	6.44 ± 0.32
After pre-cooking	1.04 ± 0.10	1.85 ± 0.23	9.29 ± 0.40	4.52 ± 0.52
After air-lift drying	0.82 ± 0.01	1.18 ± 0.14	6.48 ± 0.18	2.89 ± 0.42
Final product	0.89 ± 0.03	1.24 ± 0.07	7.15 ± 0.85	2.66 ± 0.38
'Scalp'	0.82 ± 0.08	1.06 ± 0.03	5.82 ± 0.48	2.28 ± 0.44

Asparagine and aspartic acid were more stable throughout the add-back process as compared to glutamine and glutamic acid. The major losses occurred at the drying steps. The 'scalp' was lower with respect to all of these amino acids.

4.3.1.3. FAA Pool Analysis by GLC

The N-HFB-isopropyl ester derivatives of potato FAA from a few steps in the add-back process (peeled raw potato, after pre-cooking, final product and 'scalp') were analyzed by GLC. The pattern of separation obtained for raw peeled potato FAA is shown in Figure 34. The FAA pattern of the other fractions was similar, and are not included here. Trace amounts of an unusual peak were observed, at higher sensitivity, following glycine. From standards it was determined that this was the position of α-aminobutyric acid (α-ABA).

4.3.1.4. GC-MS Identification of γ-Aminobutyric Acid (GABA) and Ornithine in Potato FAA Pool

The presence of GABA and ornithine in the FAA pool of the potato was first identified by GLC retention times, by comparison of the chromatogram of derivatized potato FAA extract with that of a similarly derivatized mixture of standard amino acids containing GABA and ornithine.

The same samples were then injected on a combined GC-MS and the mass spectra shown in Figures 35 and 36 obtained. The schematic fragmentation pattern is also given in the figures. The spectra from the potato extract and the standard mixture are identical within the limitations of the system.

The GABA spectra showed only small variations in relative

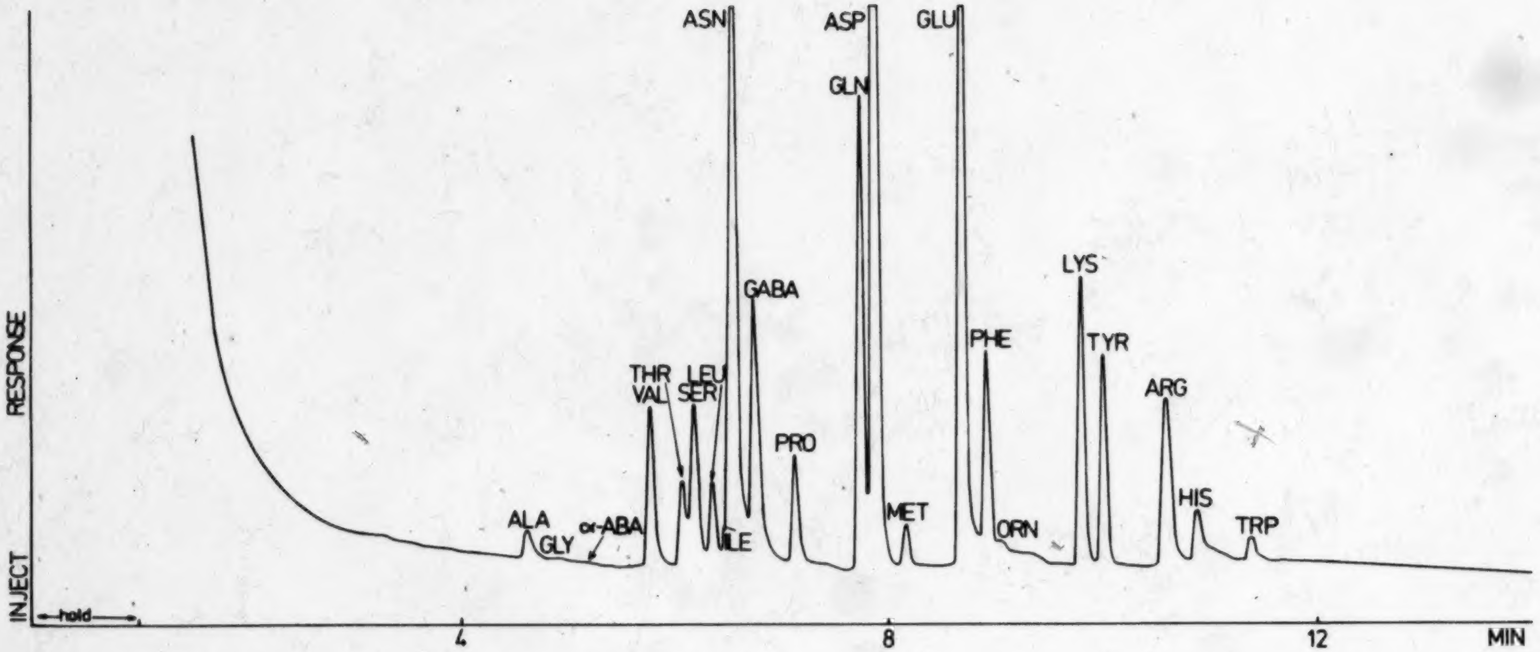


Figure 34. Gas-chromatogram of N-HFB-isopropyl ester derivatives of cation exchange purified potato FAA extract from peeled raw potato. Representing approximately 0.5 mg potato (dry weight). Temperature program: 70°C 1 min, then temperature programming at 20°C/min to 225°C and hold at 225°C.

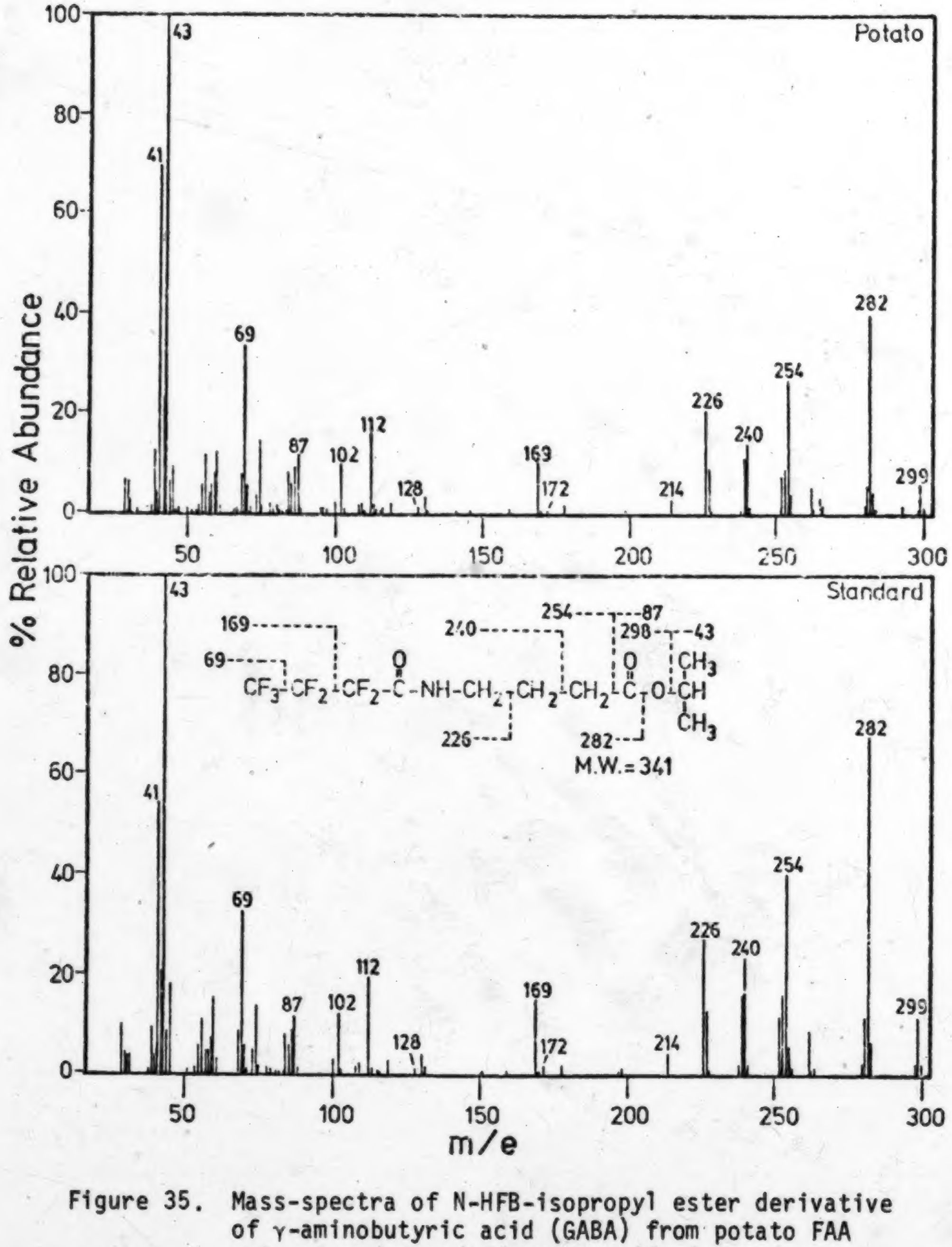


Figure 35. Mass-spectra of N-HFB-isopropyl ester derivative of γ-aminobutyric acid (GABA) from potato FAA extract and standard amino acid mixture.

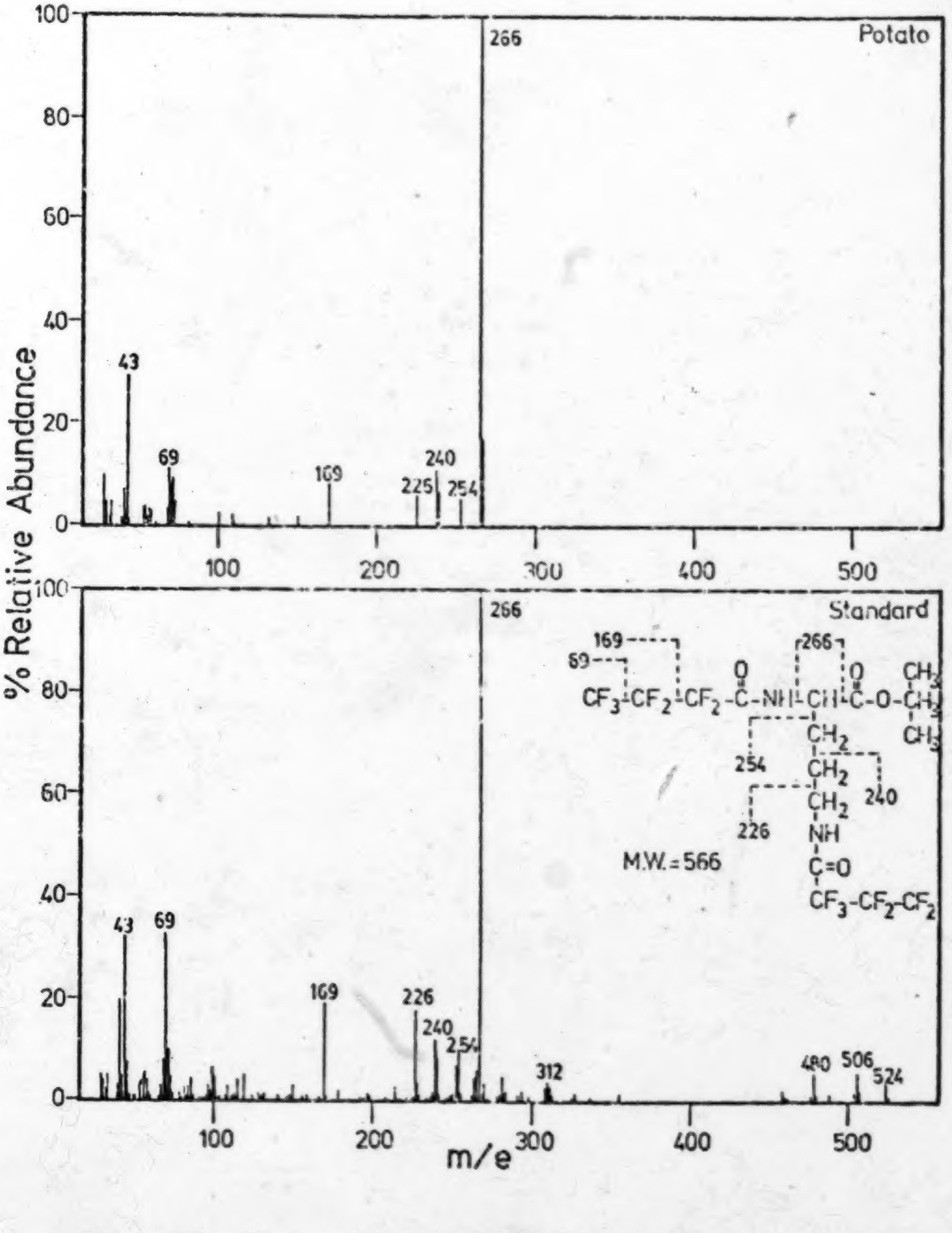


Figure 36. Mass-spectra of N-HFB-isopropyl ester derivative of ornithine from potato FAA extract and standard amino acid mixture.

intensities of the ions which are explainable in terms of the sample pressure change in the ion source during the 3-5 sec required for mass spectra scan of the sharp GLC peak. The ornithine mass spectra were somewhat less similar, because in addition to the effect of sample pressure changes, the FAA extract contained such small amounts of ornithine that some of the low abundance ions, including all those in the high mass region, fell below the detection limits of the mass spectrometer. The fragmentation patterns of both GABA and ornithine were similar to those reported for the trifluoroacetyl-*n*-butyl ester derivatives (Leimer *et al.*, 1977).

4.3.1.5. Amino Acid Composition by GLC

There were changes in the relative amounts of the amino acids during the A-B process. RMR values used for quantitative determination of FAA in the potato are given in Table 10. The presence of ornithine in potato FAA and the overlap of norleucine with γ-aminobutyric acid (GABA), which is also present in the potato, prevented the use of either one of them as an internal standard. Hence, the data obtained from the GLC analysis on a mole % basis were used in combination with the total FAA determined by the ninhydrin method, after substracting the μmoles of amides and corresponding amino acids. 'Scalp' samples were used to examine this procedure of calculation because of the low ornithine content present. Standard ornithine was added and samples analyzed in the usual way. The results obtained were very similar to the calculated values. The quantitative changes in individual amino acids on a μmole/g dry weight basis are given in Table 17. The decrease in total FAA (Table 15) was reflected by a decline in most of the amino

Table 17. Potato ('Netted Gem') free amino acid composition during granule production by the add-back process.

Amino acid \ Processing step	Peeled raw potato	After pre-cooking	Final product	'Scalp'	'Scalp'*
	µmole/g dry weight				
Ala	3.45^{b}	3.14^{b}	1.54^{a}	5.69^{c}	4.65
Gly	1.62	+	+	+	+
Val	11.67^{b}	4.02^{a}	3.28^{a}	5.80^{b}	5.07
Thr	5.06^{b}	1.74^{a}	0.76^{a}	1.75^{a}	1.13
Ser	8.18^{b}	3.39^{a}	2.08^{a}	2.56^{a}	2.35
Leu	3.14^{b}	1.74^{a}	1.16^{a}	1.29^{a}	1.24
Ile	+	+	+	+	+
GABA	31.74^{a}	30.23^{a}	33.25^{a}	24.88^{a}	24.56
Pro	4.90^{b}	5.19^{b}	1.84^{a}	2.72^{a}	2.62
Met	2.18	1.00	+	+	+
Phe	6.62^{c}	3.72^{b}	$2.42^{a,b}$	2.23^{a}	2.01
Orn	1.00	1.23	+	+	
Lys	9.50^{c}	$8.43^{b,c}$	5.23^{b}	3.32^{a}	3.03
Tyr	6.33^{b}	7.00^{b}	$3.87^{a,b}$	3.83^{a}	3.57
Arg	14.71^{c}	8.16^{b}	8.79^{b}	2.30^{a}	2.13
His	3.97^{b}	3.62^{b}	4.92^{b}	1.65^{a}	1.63
Trp	1.95^{a}	1.70^{a}	0.99^{a}	2.89^{b}	2.76
Total	116.02	84.31	70.13	60.91	56.75
% EAA**	26.1	20.00	19.9	18.7	16.8

*Average of duplicate analyses using ornithine as an internal standard.

**Percent essential amino acids of total FAA pool.

+Detected in small amount.

(a,b) A common letter in a row of means indicates that there is no significant difference at 1% level (Duncan's test).

acids during the process. GABA was the only amino acid occurring in large amounts which did not change significantly during the process. The non-significant changes in histidine and tryptophan is mainly attributed to the large experimental error involved in their measurement, as small tailing peaks. Duncan's test showed a significant (1% level) decrease in some amino acids (valine, threonine, serine, leucine, phenylalanine and arginine) at the pre-cooking step, which indicates that leaching of FAA to the cooking medium was indeed the major factor in the loss of FAA during the A-B process. The major losses of alanine, proline, lysine and tyrosine occurred at later stages in the process, where loss due to leaching was unlikely and the heat applied in the drying steps probably played the main role in their degradation.

The general composition of potato FAA pool showed that the amides, their corresponding amino acids, GABA, valine and arginine comprised approximately 80% of this fraction. The other 15 amino acids consisted of the remainder (~20%). Methionine and ornithine were detectable but not measurable at certain stages (+, Table 17). Isoleucine was present at all steps analyzed but was poorly resolved from the large peak of asparagine (Figure 31).

The significant loss at the pre-cooking step was also reflected in a decrease in % EAA (on a molar basis). Tryptophan was included in the calculation in addition to the other essential amino acids used in Tables 3 and 4. Isoleucine peak was not quantitated, therefore it was excluded from % EAA calculation.

Beyond the pre-cooking step, there was little change in % EAA (Table 17). The 'scalp' seemed to represent a different population of granules, where most of the amino acids were present in lower concentrations. Alanine and tryptophan were highest in this by-product. It was found different from the final product also in regard to % EAA, 18.7 and 19.9, respectively (Table 17).

It appears that the FAA pool of the potato is composed mainly of amides and their corresponding amino acids. Therefore, the amino acid balance in this pool is inferior to that of potato proteins. The critical step in the add-back process in terms of nitrogen and amino acids losses was the pre-cooking where, probably due to leaching of proteins and amino acids, the major losses occurred.

5. GENERAL DISCUSSION AND CONCLUSIONS

In the course of this research two distinct areas of endeavour were followed. The first was the development of systems and methodologies for the analysis of amino acids by gas-liquid chromatography (GLC). The second area was the application of the systems and methods to the analysis of the free amino acid pool of the potato, in order to evaluate the effects of processing during production of potato granules. As well, potato proteins and amino acids were analyzed and the results used to characterize the major proteins of the potato.

As a result of this dual approach, the discussion section is best presented in two parts. Part I (5.1) will deal with analytical aspects of the results and Part II (5.2) will deal with the results which pertain to the potato.

5.1. *Methodology*

5.1.1. Gas-Liquid Chromatography of Amino Acids

Amino acid analysis, employing ion-exchange chromatography was used to quantitate the amino acids of the whole potato (Tables 3, 4 and 5) and in water soluble and insoluble fractions (Table 7). This method is automated and well established (Blackburn, 1978b).

Gas-liquid chromatography offers the obvious advantages inherent in the refined separation possible at high sensitivity, small sample size required and in the speed and simplicity of the method. The availability of GLC instrumentation at low cost, the versatility of the instrument compared with specialized amino acid analyzer and the relatively simple means of combining the analytical system with mass-spectrometry for identification of unknown substances made this technique attractive. However, amino acids are not sufficiently volatile to permit direct analysis and must therefore be converted into volatile derivatives. Methods utilizing the acylalkyl ester type of derivative of amino acids have been shown to have potential for quantitative, reproducible amino acid analysis (Coulter and Hann, 1971). Therefore, this type of derivatives was used in this work. The derivative formed, N-heptafluorobutyryl-isopropyl ester (Figure 27), was reported for the first time during this work (Golan-Goldhirsh and Wolfe, 1979b). Isopropanol used as the esterification agent is more volatile than the normal alcohol (Brown, 1975). Hence, a potential for faster elution time was assumed. It was also shown that isopropyl esters of amino acids were relatively more stable to heat (Jakubke and Jeschkeit, 1977). The heptafluorobutyric anhydride (HFBA) was used as the acylating reagent because it is a strong acylating agent similar to trifluoroacetic anhydride (TFAA), but it forms more stable derivatives (Hušek and Macek, 1975). Based on these facts the potential of the N-HFB-isopropyl ester derivative of amino acids was studied. Complete analysis of 20 protein amino acids was obtained within 11 to 14 min at a temperature programming rate of 20°C/min (Figure 28a). The analytical potential of this derivative was further exploited in the identification of γ-aminobutyric acid (GABA) and ornithine in the free amino acid pool extract of the potato (Figure 34). The GC-MS system enabled the rapid direct identification of these amino acids in the potato extract. The identification was achieved by comparison of the fragmentation pattern of the derivatized amino acids in question in the potato extract to the pattern of the same amino acid derivatives in a standard mixture (Figures 35 and 36).

The resolution of most of the amino acids was almost complete, except for the group of threonine, serine, leucine and isoleucine. Reduced temperature programming rate (8°C/min) did not substantially improve the resolution in this group of amino acids (Figure 28b). The reproducibility of the pattern of separation was demonstrated by comparison of RRT values of standard amino acids and protein hydrolysates (Tables 11 and 12). In view of the large quantity of amides (asparagine and glutamine) in the potato FAA pool, it was of interest to examine the separation of the amides by the GLC method. It was shown that degradation of the amides had occurred during derivatization, as two peaks were obtained for each amide (Figures 32a,b). Derivatization in a mixture of amino acids produced a similar result. The glutamine peak was well resolved from aspartic acid but asparagine overlapped most of the isoleucine peak (Figure 33). It was concluded that at the present state of development of the method the amides cannot be determined quantitatively. It might be possible to obtain a single peak response of the amides by varying the derivatization conditions, as was reported by Hediger *et al.* (1973), using the N-trifluoroacetyl-*n*-butyl derivative (TAB). However, the complexity of this approach discouraged further research in this direction.

Quantitative determination of amino acids using the GLC method was based on the relative molar response (RMR) of each amino acid. This value was calculated relative to the ornithine molar response.

It appears from the low RMR values, especially of the more volatile derivatives of alanine, glycine and valine, that losses probably occur at the drying steps involved in the derivatization procedure. This drawback was experienced with other acylalkyl derivatives (Darbre and Blau, 1965 and Zanetta and Vincendon, 1973). The low RMR value of isoleucine, 0.461 (Table 10), was probably due to the slower esterification rate of isopropanol as a secondary alcohol (Brown, 1975). The long side chain on isoleucine could also be involved in steric hindrance in the esterification reaction. It was shown by Roach and Gehrke (1969) that by increasing the esterification time higher RMR of N-trifluoroacetyl-*n*-butyl isoleucine was obtained, but some degradation of other amino acids, especially tryptophan, occurred. More research on derivatization conditions is needed in order to optimize the RMR of isoleucine and other amino acids in a mixture. Therefore, in order to obtain quantitative amino acid analysis in protein hydrolysates or in the free amino acid extract of the potato, it was necessary to determine the RMR of standard amino acids and unknown samples under identical conditions. Characteristic RMR values used for quantitative determinations are given in Table 10. The average deviation was 9.5%, which is relatively high compared to that obtainable by ion-exchange chromatography. It is believed that automatic integration techniques can reduce this error considerably (Gill, 1973).

The GLC method was examined by comparison to the established ion-exchange chromatography (IEC) of amino acids. The same protein hydrolysates were analyzed by both methods. BSA analyses by both methods yielded virtually the same results (Table 13), but the GLC method was less accurate, mostly due to the lack of precision in the manual integration technique. Casein analyses by both methods gave similar results (Table 14). The differences which were apparent could probably be attributed to impurities in the casein preparation (Figure 31). The quantitative determination of amino acids in the free amino acid extract of the potato will be discussed later (see 5.2.).

The methodology employed in this work was based on recent developments in the GLC of amino acids. The analytical and quantitative reproducibility and accuracy of amino acid determinations using the N-heptafluorobutyryl-isopropyl ester derivatives was studied, and was shown to have significant potential. The method is based on a single column mode of operation; all derivatization steps are carried out in the same reaction vial and the esterification reagent preparation was simplified according to Felker and Bandurski (1975) and Pearce (1977). The accuracy of the method has still to be improved. Further study of derivatization conditions combined with an improved integration system will increase the accuracy of quantitative determination of amino acids by this method.

An added development while setting up the gas chromatographic system was a unique glass column connector for GC (Figure 9) (Golan-Goldhirsh and Wolfe, 1979a). The common method of connecting glass columns to a GC relies on the standard swagelok brass fittings plus o-rings or special ferrules (Rowland, 1973). Ordinary rubber o-rings will not endure high oven temperatures and deterioration usually results in severe leak problems. The column may slip out of the fittings and break, or degradation products of the rubber may produce ghost peaks or background noise or drift. Graphite or Vespel ferrules, although more heat resistant, are more expensive. In the course of this work on the separation of amino acid derivatives by GLC, the above limitations were experienced and, as a result, the new device was developed. There are several advantages to this device.

1. An all glass column, avoiding interference of injected sample with metal ions, hence preventing degradation of certain amino acid derivatives (Lamkin and Gehrke, 1965; Stalling and Gehrke, 1966).
2. It is easy to handle and easy to assemble in the GC without leaks.
3. Solves the problem of slippage and breakage due to high temperature programming, especially with hanging heavy glass columns in GC ovens such as the Varian Aerograph 2100 or Bendix 2500, and others.
4. Inexpensive, and can be reused.

This connector was used in this work and was found very useful. It is hoped that this simple device will aid chromatographers in a wide variety of situations.

5.1.2. Amides Analysis

The amides (asparagine and glutamine) were present in large quantities in the potato free amino acid (FAA) pool (Table 16 and Figure 34). Their quantitative determination could not be obtained by the GLC method as described in Section 5.1.1. Separation of the amides by IEC is also problematic as they appear as one unresolved peak under the usual analytical conditions (Figures 21 and 22). Therefore a procedure for their determination was developed. Potato FAA extract was analyzed on the IEC column in the usual way and the original (assuming no conversion of amides to the corresponding amino acids during extraction) aspartic and glutamic acids were determined. The unresolved peak containing both amides as well as serine and threonine (Figure 22) was collected in the column effluent. The fraction collected was hydrolysed with ~12N HCl, in order to completely convert the amides to the corresponding amino acids, and then rechromatographed (Figure 23). High recovery of the acids was obtained in these hydrolytic conditions (Table 9). The amides content in the FAA extract was then determined from the response of the corresponding aspartic and glutamic acids (Table 16). In the usual method of amides determination in proteins, a hydrolytic step is also involved, but the amides are determined by calculation of the difference in response of aspartic and glutamic acid in the sample before and after hydrolysis (Benson *et al.*, 1967). Alternatively ammonia released upon hydrolysis is measured, but the recovery of ammonia on the IEC system is generally not quantitative (Scoffone and Fontana, 1970), and can be due also to degradation of other amino acids (in particular serine and threonine). In the procedure proposed by Talley *et al.* (1964), more steps were involved and an assumption was made of complete absence of glutamine in the peak representing asparagine. This assumption may not be valid. The procedure developed for the results reported in this thesis has the advantage of direct quantitative determination of the amides in the form of their corresponding amino acids.

The instability of the amides, especially glutamine, is well known (Leach, 1953). Glutamine was referred to as an "easily hydrolysable amide" by Steward and Street (1946). It was noted during this work that the amides degrade partially upon storage in 0.1N HCl.

Therefore, a preliminary study of the effect of common storage temperatures on their stability was undertaken. It was shown that most of the degradation occurred at room temperature (Figures 24, 25 and 26). As was expected, glutamine degradation was faster than that of asparagine. The amount of glutamic acid formed at room temperature was much lower than the amount expected, based on the losses in the glutamine. Therefore, it is possible that some other product is formed. This may be pyrrolidonecarboxylic acid, which has been shown by Greenstein and Winitz (1961) as a likely cyclization product. At refrigeration and freezer temperatures degradation was slower and occurred within the first 5 days of storage. After that no changes were observed. In a mixture of the amides (Figure 26) the initial degradation was extended over 8 days and the combined degradation appeared somewhat smaller.

In view of the instability of the amides and possible degradation during extraction procedures (McKerrow and Robinson, 1971) on one hand, and the high content of these compounds in potato FAA pool as well as in other plants (Steward and Durzan, 1965), it is of importance to more thoroughly study the amides stability and quantitative determination.

5.2. *Potato Proteins and Amino Acids*

5.2.1. Proteins Fractionation

Total potato nitrogen was fractionated by dialysis into dialyzable and non-dialyzable nitrogen. The non-dialyzable nitrogen (proteins) was 45.4% and 37.5% of total nitrogen in outer and inner layers of the tuber, respectively (Figure 11). Total nitrogen in the outer layer (2.2%) of the tuber was significantly higher than in the inner layer (1.6%). These two observations are of importance from the analytical point of view. The wide range of values for nitrogen reported in the literature is usually attributed to the effect of growth conditions, fertilization levels, dry matter content, etc. But the nitrogen distribution in the tuber was overlooked to a large extent (Talburt *et al.*, 1975). In an early report Cotrufo and Levitt (1958) first indicated that it is necessary not only to investigate the inner layer but also the outer layer of the tuber in relation to protein metabolism in the potato. In a later report Ashford and Levitt (1965) showed that at all times in storage at 3°C or after storage at 3°C and transfer to 26°C for 10 days before analysis the ratio of protein nitrogen to NPN is higher in the outer layer than in the inner layer of the tuber. Recently, Weaver *et al.* (1978) reported on differences among different parts of the tuber. They compared the bud-end, stem-end and the pith for total and free amino acid nitrogen and other constituents. They found significant differences among different parts of the tuber in regard to these constituents. Pith tissue in all cultivars tested had more nitrogen than did either end of the tuber. Total nitrogen content was similar at both ends of the tuber. Johnston *et al.* (1968) reported that the stem-end tissue contained more total nitrogen than did the bud-end tissue. These reports and the results obtained in this work regarding the OL and IL of the tuber indicate that significant variations in determination of nitrogen content could be introduced by lack of attention to the sampling position within the tuber.

The proteins (non-dialyzable fraction) were further fractionated according to solubility (Table 6). It was shown that the albumins and globulins constitute the major portion of potato proteins (approximately 80%). This was also shown by others (Groot *et al.*, 1947; Lindner *et al.*, 1960; Kapoor *et al.*, 1975a). The 0.2% NaOH soluble glutelins constituted approximately 16% of total potato proteins and the prolamines and the acidic glutelins an almost negligible amount.

There is still disagreement about the relative amount of the albumins and globulins fractionated by the different procedures. The procedure used in this work yielded 53.1% and 46.4% albumins and 27.6% and 36.0% globulins in outer and inner layers, respectively (Table 6). Kapoor *et al.* (1975a) obtained a similar pattern for whole potato tuber proteins, employing a similar fractionation method based on Nagy *et al.* (1941). A different relative amount of albumins and globulins was obtained by Kapoor *et al.* (1975a) employing the method of fractionation suggested by Lindner *et al.* (1960). That procedure is based on extraction of the albumins and globulins together in dilute salt solution, then separation of the protein fractions by differential precipitation with ammonium sulfate. The low salt soluble fraction (tuberin) comprised 71.3% and the water soluble fraction (albumins) was 6.6% of total potato proteins. It appears that the procedure used here yielded better separation between albumins and globulins. Kapoor *et al.* (1975a) suggested that perhaps up to 40% of the tuberin obtained by Lindner's procedure may be albumins. This appeared to be possible since the results of both methods for albumins and globulins were similar and in accordance with results obtained here. Kapoor *et al.* (1975a) suggested that there may be 46-48% albumins and 26-30% globulins. The results obtained here confirmed this estimation. Levitt (1951) and Nakasone *et al.* (1972) separated potato proteins into nearly equal fractions of albumins and globulins. These results also support the general pattern obtained here, where the albumins constituted approximately half of the tuber's proteins and the other protein fractions the other half.

In further fractionation of potato proteins the water soluble and insoluble fractions were extracted with the solution suggested by Nakasone *et al.* (1972). Column chromatography on Sephadex G-150 revealed that the water soluble proteins were composed of 4 major fractions (Figure 19). Nakasone *et al.* (1972) obtained 3 fractions on Sephadex G-100. The high molecular weight fraction which came off the G-150 column in the void volume represents high molecular weight proteins, >160,000 (A, Figure 19). The two intermediate protein fractions (B,C Figure 19) were estimated to be 76,000 and 20,000, respectively. Similar molecular weight estimates were obtained by Nakasone *et al.* (1972), 80,000 and 25,000. A fourth fraction (D, Figure 19) of low molecular weight (<13,000) might represent small protein subunits and peptides separated from large protein molecules during the extraction steps. The pattern of separation remained constant during tuber growth, except for changes in the relative amounts of the fractions (Table 8). The water soluble proteins of the OL of the tuber at maturity showed a similar pattern of separation. The 76,000 fraction was significantly smaller and the small molecular weight one was especially big, probably due to non-protein compounds, as was indicated by lower protein content in this fraction as measured by Lowry's method (Appendix 2). The large molecular weight fraction was relatively the largest in the mature tuber (Table 8 and Appendix 2) which may indicate a build-up of large protein molecules as storage material.

Electrophoretic separation of water soluble and insoluble proteins on SDS-PAGE after SDS,DTT treatment revealed that the major potato protein subunits in both fractions are rather small, having molecular weights in the 10,000 and 30,000 range (Figure 12). This is in accordance with results obtained by Stegemann *et al.* (1973). They showed that the small molecular weight subunits were apparent only after treatment with a reducing agent (mercaptoethanol), which indicated that some protein subunits are linked by S-S bridges. Electrophoretic analysis on different gel concentrations (8.5% and 10%) did not resolve more protein bands (Figures 13 and 14).

Thin layer isoelectric focusing (TLIF) of the water insoluble fraction showed 8 protein bands (Figure 16). The dominant bands were focused in pH 8.5 region. Amino acid analysis of this fraction (Table 7) showed higher content of basic amino acids, which partly explain the high isoelectric points of these proteins. In an early investigation of the isoelectric pH of protein fractions (Groot *et al.*, 1947), it was first found that the globulins (tuberin) have higher isoelectric points than the albumins (tuberinin). It will be of interest in this regard to verify the amides content of these proteins, as amides might be an important factor in the explanation of the high isoelectric pH of this fraction. It was shown by Stegemann *et al.* (1973) that the degree of amidation controls to a large extent the charge of potato proteins. Thirteen protein bands were resolved in the water soluble fraction (Figure 15), covering a wide range of isoelectric pHs (4.5-8.5). The TLIF method of analysis revealed that the fractionation of the proteins according to solubility characteristics is not complete. According to electrophoretic patterns it appears that a residual amount of the major protein bands in the water insoluble fraction appeared also in the water soluble fraction and vice versa (Figures 15 and 16).

The pattern of separation of sap proteins (Figure 17) contained both the protein bands of the water soluble and water insoluble fractions and additional proteins which were probably better preserved in the faster extraction process because of the shorter time interval between separation from the intact tuber and application to the gel. This method was probably the best for preservation of the proteins in their native states. Hence, a better approach to potato proteins fractionation could be based on the extraction of sap proteins according to the method of Stegemann *et al.* (1973) (as was used here), followed by separation of total sap proteins using TLIF on granulated gel on a preparative scale according to Radola (1974). When using TLIF on a preparative scale, the separated protein bands should be easily eluted from the granulated gel (Radola, 1974). Each fraction can then be further characterized according to solubility or according to other physicochemical methods.

The TLIF pattern of sap proteins from the inner layer, outer layer (excluding 'skin') and whole tuber was virtually the same (Figure 18). Stegemann *et al.* (1973) have also reported that the bud-end and stem-end as well as other cuts from the peripheric to the center region of a peeled potato displayed similar protein spectra. The 'skin' itself had some proteins not matching with the usual electrophoretic pattern of the tuber.

5.2.2. Proteins and Amino Acids Content of the Potato Tuber

Potatoes are generally not considered to be a significant dietary source of protein, in spite of a few reports which demonstrated that the potato, in combination with other proteins, is an excellent dietary protein source (Markakis, 1975). When considering the nitrogen content of the potato, it must be remembered that more than 50% of the tuber nitrogen is in a non-protein form (Figure 11). This does not necessarily imply lower nutritional quality, but probably higher susceptibility to losses during home cooking or industrial processing by mere leaching to the cooking media.

An accepted concept is that "protein nutrition is essentially amino acid nutrition" (Oser, 1951). Consequently, the amount of free amino acids in the non-protein nitrogen (NPN) fraction of the potato has been considered by Woodward and Talley (1953), Hughes (1958), Talley *et al.* (1964) and others as of nutritional importance.

The average total nitrogen in the different potato cultivars examined in this study was 1.8% on dry weight basis (Table 1). This value, although within the range reported in the literature (Talburt *et al.*, 1975), is relatively high and may be a result of high nitrogen fertilization. Increase in total nitrogen upon nitrogen fertilization has been reported (Mulder and Bakema, 1956; Hoff *et al.*, 1972; Eppendorfer, 1978). It was shown to increase mainly the NPN and less the protein nitrogen. This was correlated with a decrease in the nutritive value of the potato evaluated by the 'essential amino acid index' value (Schuphan, 1970).

There was an apparent inverse relationship between dry matter content and % nitrogen in dry matter, but % nitrogen on fresh weight basis was the same in the different cultivars (Table 1). These results may be interpreted that the nitrogen did not vary appreciably in mature tubers of different cultivars and the apparent inverse relationship between dry matter content and total nitrogen content on dry weight basis is the result of an increased content of some other dry matter constituent, presumably starch. These relationships were shown for tubers of 'Katahdin' cultivars of different dry matter contents (Talley *et al.*, 1961).

The expression of total nitrogen of the potato as total protein by using the 6.25 factor is commonly used, but it is still subject to further investigation in relation to potato proteins. It was recently reported by Desborough and Weiser (1974) that potato proteins contain 13.5% nitrogen, making 7.4 a more appropriate factor to use. But, no account for amide nitrogen and degradation of other amino acids upon hydrolysis was made. Therefore, more research on nitrogen content in potato proteins and amino acids is needed in order to verify the appropriate factor of conversion for micro-Kjeldahl nitrogen to total potato proteins.

Variability in total nitrogen content due to location and condition of growth etc. has been reported extensively in the literature. This variability was reflected also in this work by the differences in nitrogen values obtained for different batches of 'Netted Gem' tuber used. In the varieties comparison (Table 1), 'Netted Gem' had 1.5% nitrogen while in the protein solubility study (Figure 11) a value of 1.6% for total nitrogen was obtained, and tubers grown in southern Alberta used in the study of the add-back process had a high value of 1.8% (Table 15).

The change in percent nitrogen content on dry weight basis during 'Netted Gem' tuber growth did not show a clear trend of change. But, percent nitrogen content on fresh weight basis showed a trend of increase (Table 2). This probably indicates accumulation of proteins as storage material in the mature tuber. High nitrogen content in mature tubers was also reported by Talburt *et al.* (1975).

Total amino acids recovered (Tables 3 and 4) were lower than total protein estimated by micro-Kjeldahl (Tables 1 and 2). This may be accounted for by losses of amides nitrogen and amino acids degradation which occur during hydrolysis in 6N HCl for amino acid analysis. In addition, there is approximately 8% of other organic nitrogen not measured by the amino acid analyzer (Markakis, 1975).

A characteristic pattern of amino acids was found in all cultivars examined (Table 3). Approximately 38.2% of the total amino acids recovered were 'essential amino acids'. The rest were 'non-essential amino acids', consisting mainly of aspartic and glutamic acids, which represent a combined value of the amino acid and the corresponding hydrolysed amide. The largest variations among cultivars appear to be related to differences in these amino acids. Methionine was present in all cultivars, but was in measurable quantity only in 'Norland', 'Alaska Red' and 'Kennebec'. This amino acid is degraded upon hydrolysis (Blackburn, 1978a), therefore its quantitative estimation should be considered cautiously. However, methionine is considered to be the first limiting amino acid of the potato (Kaldy and Markakis, 1972). γ-aminobutyric acid (GABA) was present in all the cultivars; it was especially high in the 'Norland'. This is a non-protein amino acid (Thompson *et al.*, 1953) and it was derived from the free amino acid fraction of the potato, where it is present in a substantial amount (Table 17).

Consideration of amino acid composition of the 'Netted Gem' cultivar in relation to harvest time (Table 4) indicated a trend of decrease in total amino acids, which is reflected by a decrease in almost all individual amino acids. The general pattern of amino acid composition was similar throughout the growing season. It seems that young tubers have higher proportion of 'essential amino acids' (42.9%) than mature tubers 35.3% (Table 4). Jaswal (1973) has shown similarly that 'essential amino acids' in the mature tuber constitute approximately 1/3 of the total amino acids of the tuber. A trend of decrease in 'essential amino acid index' with increasing tuber size was also shown by Shuphan (1970). The direct nutritional implications of this trend might be limited, because small immature tubers are usually not consumed, but as stated by Schuphan (1970) this observation is of interest to both nutritionists and plant breeders.

Peeling losses in home preparation or in industrial processing are large and depend on tuber size (Tables 1 and 2), and the process used (Huxsoll and Smith, 1975). The hand peeling used in this work amounted to an average 10.2% loss of fresh weight of mature tubers. Peeling losses are of economical concern to the processors (Powers *et al.*, 1977; Muneta and Jennings, 1978). An increased yield of chips made of unpeeled potatoes was reported recently (Shaw *et al.*, 1973). They found no significant differences between chips made of peeled and unpeeled potatoes in terms of flavor, appearance, shelf life and stability of oil. Another concern relates to peeling losses in the waste generated and the environmental problems associated with disposal. Methods of recovery of potato proteins from potato processing waste effluent were reported recently by Knorr (1977). It was shown here that the OL (peel) of the tuber contained higher percent nitrogen (2.2%) than the IL (1.6%) (Figure 11). A larger portion of protein

(45.4%) was in the OL than in the IL (37.5%). Percent 'essential amino acids' in the OL was higher, 38.3% than in the IL, 35.3% (Table 5). Schuphan also reported higher 'essential amino acid' content in the OL of the potato. This is probably due to the more favorable amino acids pattern of potato proteins in comparison to the free amino acid pool. It was shown that the free amino acid pool has a low percent of 'essential amino acids' (Table 17). The low proportion of 'essential amino acids' in the free amino acid pool of the potato was also shown by others (Hoff *et al.*, 1971; Jaswal, 1973). The higher protein content of the OL (peel) and the more favorable amino acid composition of potato proteins means that the peeling losses should be of nutritional concern, in addition to the economic concern and the waste problem.

5.2.3. Effect of the Add-Back Process on Total Potato Proteins and Free Amino Acids

The information in the literature on processing effects on potato proteins and amino acids is scanty (Jaswal, 1973). There are no available reports on the effect of the add-back process on total potato proteins and free amino acids. In view of the important role that potato granules production has, especially in Alberta, it was important to conduct this investigation. The methodology developed in this work for amino acids and amides analysis was used.

The general composition of total proteins and free amino acids of the potato during the add-back process (Table 15) showed that a significant drop in both occurred at the pre-cooking and the mash-mixing steps. Beyond these stages in the process there was a continuous slight decrease in total nitrogen which was not significant. There were also non-significant changes in the FAA in later stages in the process. However, the slight increase in the FAA fraction at the air-lift step, although not significant, might reflect an increase in the extractability of FAA due to changes in the physical properties of the granules. This has to be further examined in relation to the efficiency of extraction with 70% EtOH at different stages of the add-back process.

The overall loss in total nitrogen amounted to 38.9% and the total loss in FAA was 36.8%. Desborough and Weiser (1974) reported a 50% loss of total nitrogen after cooking for 30 min in boiling water. The Vauxhall pre-cooking step involves cooking for 15-20 min at approximately 70°C, and resulted in a 16.7% loss. Thus, it can be assumed that temperature and time of cooking may have a critical effect on the extent of the loss.

The pre-cooking step seems to be the critical stage in the add-back process. The losses at this stage are most likely due to leaching of solutes into the cooking water. The losses in the mash-mixing step probably reflect a few factors such as the cooling in water and steam cooking stages, between the pre-cooking and the mash-mixing, and the high temperature in the mash mixer (~50°C). As well, the addition of "add-back" granules with lower nitrogen content is probably also reflected in the decrease of total nitrogen at this step.

Based on the average molecular weight (140.15) of the amino acids used in construction of the standard curve for total amino acid determination (Figure 6) and total FAA determined, peeled raw potato with 249.53 µmole/g (Table 15), the FAA content of the potato is calculated to be 34.97 mg/g. It represents 31.1% of total potato proteins (N x 6.25)

(Table 15). A similar calculation for FAA (Table 17) excluding the amides, glutamic and aspartic acids shows that the FAA comprises only 14.4% of the total protein of the potato (N x 6.25) (Table 15). This result is similar to the value of 15% (including glutamic and aspartic acids) suggested by Markakis (1975). It thus can be concluded that the FAA pool in absolute terms contributes only a small proportion to total potato proteins. The major part of the FAA pool is composed of the amides and their corresponding amino acids, 53.5% of the total pool in peeled raw potato (on molar basis). Hence, the losses in this fraction, although significant, are only part of the picture. The other part is related to the changes in protein amino acids. This aspect requires further research in the future.

The fact that the 'scalp' was not significantly different from the raw potato tuber in regard to total protein content (Table 15), on one hand, and the significantly lower content of FAA on the other hand, may indicate two things. 1. The 'scalp' is probably composed more of protein nitrogen than of FAA. 2. In view of the fact that the 'scalp' is composed mainly of peel pieces and attached starch and granules, it is suggested that the 'scalp' fraction is similar to the OL of the tuber. Thus, the higher nitrogen content of the 'scalp' is corroborative with our previous observation of higher nitrogen content in the OL compared with the IL of the tuber. The 'scalp', which is discarded from the granules is used for animal feed. Nutritionally, it might have an even better amino acid balance than the potato granules because of the higher protein content.

The characteristic pattern of separation of the FAA pool of the potato (Figure 31) shows that the amides (asparagine and glutamine), aspartic acid and glutamic acids and γ-aminobutyric acid are the dominant amino acids, as was shown by Synge (1977) and Davies (1977) and others. The other amino acids appeared in relatively very low amounts in the FAA pool of the potato. Asparagine and glutamine are the major amino acids in this pool (Table 16). Asparagine was less degradable during the add-back process in comparison to glutamine. This is corroborative with our previous observations regarding the stability of the amides (see Section 5.1.2.).

The quantitative determination of the individual amino acids (Tables 16 and 17) showed that the pattern of amino acids of the FAA pool is inferior to that of the potato proteins. Percent 'essential amino acids' of the peeled raw potato, 26.1% (Table 17), was markedly lower compared to that of the total proteins, 38.2% (average of the different cultivars). The low relative amount of the 'essential amino acids' is mainly due to the extremely high percentage (53.5%, calculated on μmole/g basis) of amides and their corresponding amino acids in the FAA pool of the peeled raw potato. Variations in the absolute values reported in the literature are due to the susceptibility of the FAA pool to the effect of conditions of growth, cultivars examined, etc. Hoff *et al.* (1971) reported that this group of amino acids in the 'Superior' cultivar constituted the bulk of the FAA pool and increased from 55% to 65% with increasing nitrogen fertilization.

The loss in total amino acids was reflected in losses of individual amino acids during the add-back process. Most of the losses occurred at the pre-cooking step. The long cooking (~20 min) in water at approximately 70°C resulted in leaching of the FAA. Significant losses were found in valine, threonine, serine, leucine and phenylalanine. The loss of alanine, proline, lysine and tyrosine was significant at later stages in the process where heating during drying played the major role in the degradation of these amino acids. Lysine is one amino acid which has been studied extensively in relation to Maillard reactions (Bender, 1971). Most of these studies were related to the loss of lysine availability in proteins, but the same type of reaction can occur with unbound lysine or any other free α-amino group. High carbohydrates content and the elevated temperatures at the drying stages are in favor of lysine-Maillard reaction. Therefore high lysine losses are expected.

Methionine, the only sulfur containing amino acid found, was also substantially degraded and was present in detectable amounts only in the final product and 'scalp'.

The loss of the 'essential amino acids' was reflected in the decrease of % EAA from 26.1% to 19.6% in the final product. However, the large decrease in the amides and corresponding amino acids which constitute the major part of the FAA pool are the major source for the losses during the add-back process on an absolute basis. In a comparable study of the effects of various processing methods on free and bound amino acids of potatoes, Jaswal (1973) showed approximately a 40% loss of total amino acid content in canning and chip making, while in drum dried potatoes approximately 20% loss was measured.

The add-back process consists of two major processing effects which adversely affected the total protein and free amino acids in the final potato granules. These stages in the process are integral parts of the process. Changing these steps might change the physical properties of the final product. Recently, a new process, the freeze-thaw process, was developed by Ooraikul (1977 and 1978). In this process losses are likely to occur mainly at the drying stages where heat treatment is used. Heating is an unavoidable step in dry potato granules production. It was shown here that loss of a few amino acids was significant at the drying steps. There was no significant changes in the other amino acids at these stages. In addition, the steam cooking is used in the freeze-thaw process in which leaching of solutes is minimized. Therefore, an assumption can be made that the freeze-thaw process will better preserve total potato proteins and amino acids.

5.3. *Conclusions*

The major conclusions are as follows:

1. Complete gas chromatographic analysis of amino acids can be obtained at high speed by using the N-heptafluorobutyryl-isopropyl ester derivatives. The method is applicable to protein hydrolysates, as well as for free amino acid extracts analysis. It will be possible to increase the accuracy of the quantitative determination by optimization of derivatization conditions and improved integration system.
2. Interfacing the GLC system with a mass spectrometer provides a tool for fast direct identification of amino acids.
3. The glass column connector developed in this work solves the difficulties encountered in connecting heavy glass columns to a GC. It is especially useful for high temperature programming.
4. The procedure for amides determination enables direct determination

of the amides from the response of their corresponding amino acids, rather than by calculation as is usually done. It might be applied to amides determination in any FAA extract. The instability of the amides has to be further investigated.

5. Approximately 60% of potato ('Netted Gem') nitrogen was found in a dialyzable form. It was shown that the FAA in this fraction are mainly composed of amides, their corresponding amino acids, and γ-aminobutyric acid. The other amino acids occur in low amounts. Therefore, the FAA fraction is nutritionally not well balanced.
6. Potato protein fractionation patterns (according to solubility), although empirical and dependent on the procedure of fractionation, showed that the albumins and globulins are the major potato proteins, approximately 50 and 30%, respectively. Incomplete separation between the water soluble and insoluble fractions was revealed by TLIF on granulated gel. This finding suggests that TLIF may have the potential for better fractionation of potato proteins. However, it was shown that the water insoluble fraction is composed of basic proteins. The water soluble fraction was composed of more proteins with diverse isoelectric points. The major protein subunits in both fractions had low molecular weights. No differences in the composition of sap proteins of the outer and inner layers of the tuber were found.
7. Nitrogen content on a fresh weight basis showed differences among cultivars on dry weight basis, indicating that the commonly used expression of nitrogen on dry weight basis might sometimes exaggerate differences between cultivars in comparative

studies. Similarly, no clear trend of change in nitrogen content during 'Netted Gem' tuber growth was observed when expressed on dry weight basis, while on fresh weight basis there was an increase in nitrogen content, indicating probably a trend of accumulation of storage proteins in the mature tuber.

8. The pattern of amino acid composition in the different cultivars, as well as in 'Netted Gem' tuber during growth, was similar. Aspartic and glutamic acids were the major amino acids. High proportion of 'essential amino acids' (% EAA) was found (average 38.2%). Methionine appeared in the lowest amount in all IEC analyses and cystine was detected only in small quantities in the extracted protein fractions, suggesting, as was shown by others, that these are the limiting amino acids of the potato.
9. Nitrogen and protein content in the outer layer of 'Netted Gem' tuber was higher than in the inner layer. % EAA was also slightly higher in the outer layer, suggesting that peeling in home preparation or industrial processing can result in a loss of nutritional quality of the potato.
10. The major losses in nitrogen and FAA of the potato were detected in the pre-cooking and mash-mixing steps, indicating that the losses of nitrogenous compounds in the process are probably due mainly to leaching of amino acids and proteins in the cooking and cooling steps. The relatively high nitrogen content in the 'scalp' suggested that this fraction is probably derived mainly from the OL of the tuber. The losses in the FAA fraction were mainly due to losses of the amides and corresponding amino acids. The small

amount of other amino acids in this fraction had only a small contribution to the total loss. Further studies of the losses in protein amino acids are required in order to achieve an improvement of the nutritional quality of the final product.

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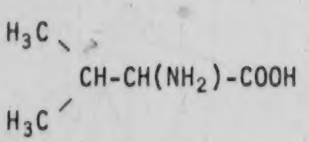
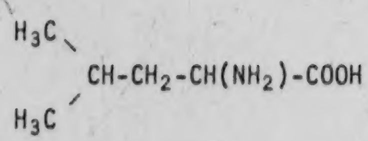
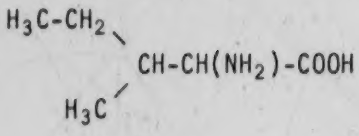
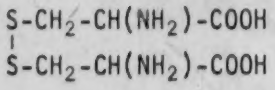
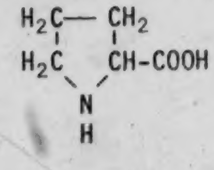
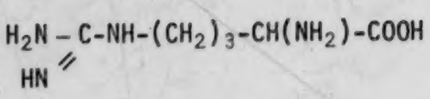
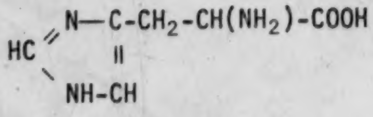
7. APPENDICES

APPENDIX I

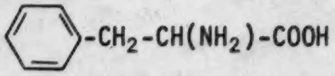
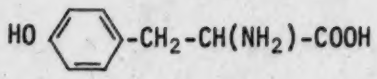
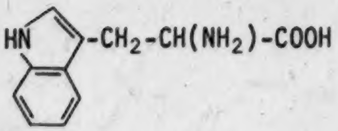
Classification of Protein Amino Acids

Class	*Name*	*Abbreviation**	*Chemical Structure*
Aliphatic neutral amino acids	Glycine	Gly	H_2N-CH_2-COOH
	Alanine	Ala	$CH_3-CH(NH_2)-COOH$
	Valine†	Val	 $\begin{array}{l} H_3C \diagdown \\ \qquad CH-CH(NH_2)-COOH \\ H_3C \diagup \end{array}$
	Leucine†	Leu	 $\begin{array}{l} H_3C \diagdown \\ \qquad CH-CH_2-CH(NH_2)-COOH \\ H_3C \diagup \end{array}$
	Isoleucine†	Ile	 $\begin{array}{l} H_3C-CH_2 \diagdown \\ \qquad\qquad CH-CH(NH_2)-COOH \\ \qquad H_3C \diagup \end{array}$
Aliphatic hydroxy amino acids	Serine	Ser	$HOCH_2-CH(NH_2)-COOH$
	Theronine†	Thr	$H_3C-CHOH-CH(NH_2)-COOH$
Sulfur-containing amino acids	Cysteine	Cys	$HS-CH_2-CH(NH_2)-COOH$
	Cystine	Cys_2	 $\begin{array}{l} S-CH_2-CH(NH_2)-COOH \\ \mid \\ S-CH_2-CH(NH_2)-COOH \end{array}$

APPENDIX I (Continued)

Class	*Name*	*Abbreviation**	*Chemical Structure*
	Methionine†	Met	$H_3C\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
Imino acid	Proline	Pro	
Basic amino acids	Lysine†	Lys	$H_2N\text{-}CH_2(CH_2)_3\text{-}CH(NH_2)\text{-}COOH$
	Arginine†	Arg	 $H_2N-\underset{\overset{\Vert}{HN}}{C}\text{-}NH\text{-}(CH_2)_3\text{-}CH(NH_2)\text{-}COOH$
	Histidine†	His	
Acidic amino acids and corresponding amides	Aspartic acid	Asp	$HOOC\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
	Asparagine	Asn	$H_2N\text{-}CO\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
	Glutamic acid	Glu	$HOOC\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
	Glutamine	Gln	$H_2N\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$

APPENDIX I (Continued)

Class	*Name*	*Abbreivation*[*]	*Chemical Structure*
Aromatic and heteroaromatic amino acids	Phenylalanine[†]	Phe	
	Tyrosine	Tyr	
	Tryptophan	Trp	

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[*] Abbreviations used in the text. On figures big letters were used.

[†] 'Essential amino acid'.

Other naturally occurring amino acids mentioned were: Ornithine, γ-aminobutyric acid, α-aminobutyric acid, norleucine, β-alanine, L-pipecolic acid and S-methylmethionine.

APPENDIX 2

Potato ('Netted Gem') water soluble protein fractions during tuber development, analyzed by column chromatography on Sephadex G-150 and measured by Lowry's method.

Fraction	A	B	C	D
Harvest date	Percent of total recovery*			
Jul 27	15.3	28.7	26.7	26.6
Aug 16	11.9	28.6	31.7	27.7
Aug 31	15.6	21.6	40.7	21.4
Sept 9	19.7	24.2	32.9	23.2
Sept 24	15.5	24.6	37.2	22.7
Oct 28**	21.3	23.4	37.7	17.6
Oct 28***	36.4	7.8	27.2	28.6

*Protein was determined on each fraction (A, B, C and D at every harvest date and is expressed as a percent of total protein recovered.

**Inner layer at maturity.

***Outer layer at maturity.

APPENDIX 3

Effect of derivatization temperature and time on RMR of N-HFB-isopropyl esters of standard amino acids*.

	Acylation 150°C/5 min						Acylation 110°C/10 min					
	Esterification temp (°C)						Esterification temp (°C)					
Time	80			110			80			110		
AA min	30	60	120	15	30	60	30	60	120	15	30	60
Ala	0.60	0.43	0.25	0.34	0.30	0.16	0.30	0.32	0.54	0.45	0.30	0.19
Gly	0.73	0.56	0.45	0.62	0.45	0.45	0.59	0.54	0.75	0.62	0.45	0.38
Val	0.44	0.60	0.44	0.29	0.38	0.26	0.22	0.27	0.56	0.26	0.38	0.29
Thr	0.62	0.67	0.66	0.39	0.51	0.50	0.39	0.46	0.72	0.41	0.51	0.48
Ser	0.60	0.59	0.60	0.43	0.36	0.49	0.53	0.57	0.70	0.48	0.52	0.48
Leu	0.77	0.80	0.66	0.76	0.73	0.58	0.66	0.67	0.87	0.63	0.71	0.51
Ile	0.37	0.49	0.54	0.12	0.29	0.31	0.20	0.26	0.55	0.20	0.33	0.33
Pro	0.92	0.79	0.70	0.94	0.74	0.60	0.98	0.87	0.96	1.11	0.74	0.52
Cys**	0.78	0.50	0.60	0.64	0.66	0.66	-	-	-	0.58	0.66	0.50
Asp	0.82	0.86	0.84	0.75	0.75	0.71	0.72	0.75	0.93	0.75	0.74	0.69
Met	0.69	0.82	0.80	0.56	0.65	0.62	0.51	0.69	0.76	0.60	0.65	0.70
Glu	0.91	0.85	0.83	0.78	0.83	0.77	0.87	0.90	0.98	0.94	0.83	0.77
Phe	1.20	1.24	1.31	1.12	1.08	1.13	1.15	1.16	1.28	1.07	1.08	1.19
Lys	0.82	0.73	0.70	0.90	0.91	0.68	0.97	0.97	1.04	0.44	0.79	0.96
Tyr	1.16	1.18	1.29	1.26	1.44	1.22	1.19	1.18	1.34	0.52	1.24	1.30
Arg	1.24	1.18	1.22	1.08	1.09	0.94	1.29	1.24	1.32	1.04	1.09	1.21
His	0.71	0.74	0.87	0.57	0.80	0.85	0.75	0.82	0.96	0.71	0.80	0.91
Trp	0.64	0.70	0.84	0.95	0.67	0.80	-	-	-	0.64	0.67	0.70
Cys_2**	1.04	1.60	1.56	1.34	1.26	1.74	0.72	0.86	1.24	0.94	1.26	1.40
Average % CV	8.5	14.3	16.8	15.4	22.3	5.9	14.9	13.0	7.1	4.4	5.7	7.6

Automatic integration used for peak area measurement. At least three replicates were run for each condition.

*Equimolar mixture of amino acids (2.5 µmole/ml).

**Cys = cysteine (1.25 µmole/ml); cys_2 = cystine (1.25 µmole/ml).